Monoclonal antibody-based Surface Plasmon Resonance sensors for pathogen detection

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ABSTRACT

A biosensor is an analytical device, which incorporates a biological sensing element integrated within a physicochemical transducer. The aim of a biosensor is to produce an electronic signal, which is proportional to the interaction of analytes with the sensing element. This means that the sensor essentially transforms molecular interactions into a digital signal, thereby making detection of analytes label-free. Biosensors are used for detection of analytes ranging from small drug molecules to food- and waterborne microorganisms as well as biowarfare pathogens.

In future farming, plant production will be concentrated at few and very large farms. In order to reduce the pesticide use, it is necessary for the farm manager to have detailed knowledge of the distribution of weeds, diseases and pests within the fields. However, field-monitoring by manual inspection is time consuming and expensive. Biosensors, that can detect and quantify specific plant pathogens and map these to defined positions within the field, would enable the farm manager to perform a precise and targeted application of pesticides and thereby reduce and optimise the use of agrochemicals. The ideal scenario for precision agriculture is to have real-time, robust and low-cost sensors, for both soil and air, which can be operated by personnel with limited or no training in plant pathology.

In the present thesis focus is put on the development of immunological sensors for detection of two model plant pathogens, *Puccinia striiformis* f.sp. *tritici*, the cause of wheat yellow rust and *Phytophthora infestans*, the cause of late blight disease in potato.

As no antibody existed against urediniospores from *P. striiformis*, mouse monoclonal antibodies (mAbs) were produced and characterised. IgM-isotype mAbs from nine hybridoma cell lines were screened for cross-reactivity against representatives from common genera. Two specific mAbs were chosen for further characterisation and used to develop a competitive ELISA (using mAb4) and a subtractive inhibition ELISA (using mAb8). The subtractive inhibition ELISA was found to be more sensitive with a detection limit of 1.5 x 10^5 urediniospores/ml. The assay setup consists of incubation of urediniospores with mAb8, removal of

urediniospore-bound mAb8 by centrifugation and quantification of the remaining unbound mAb8 by rabbit anti-mouse IgM antibody. The remaining free mAb8 is thereby related to the initial cell concentration. Assay performance was investigated by cross-reactivity studies against other rust fungi. Cross-reactivity was found with *Puccinia recondita* and *Puccinia hordei*, suggesting that the ~ 39 kDa mAb8-antigen might be a conserved structural component in the surface of *Puccinia* species.

The subtractive inhibition assay was further developed for label-free detection using a Surface Plasmon Resonance sensor. The polyclonal anti-mouse IgM was immobilised on a sensor surface and used for capture and quantification of mAb8. Optimal regeneration conditions were identified and 20 mM HCl effectively regenerated the surface. The assay had a similar sensitivity as the ELISA with a detection limit of 3.1×10^5 urediniospores/ml. *P. striiformis* was furthermore detected in a mixture with the rust species *Melampsora euphorbia*, which underlined the specificity of the sensor.

A Surface Plasmon Resonance sensor was further developed for detection of *P. infestans* sporangia. An existing *Phytophthora* genera mAb (phyt/G1470) was found to be highly specific when tested for cross-reactivity against spores from ascomycetes, deuteromycetes and basidiomycetes in a subtractive inhibition ELISA. The subtractive inhibition assay was incorporated in a Surface Plasmon Resonance sensor and optimal assay and regeneration conditions were identified. Calibration curves were generated and a detection limit of 2.22×10^6 sporangia/ml was achieved. The assay analysis time of 75 minutes is superior to existing immuno- and nucleotide-based assays for *P. infestans* detection.

In conclusion, the results presented in this thesis describe the first use of Surface Plasmon Resonance immunosensors for plant pathogen detection and represent a first step towards the implementation of plant pathogen immunosensors on-site.

DANSK RESUME

En biosensor er en analytisk anordning, hvori et biologisk detektionselement er integreret i en fysisk-kemisk transducer. Biosensoren producerer et elektrisk signal, som er proportionelt med analyttens interaktion med detektionselementet. Dette betyder, at sensoren omdanner molekylære interaktioner til et elektronisk signal, hvilket gør det muligt at detektere analytter uden brug af mærkninger, som f.x. fluorescens. Biosensorer bliver brugt til detektion af forskellige analytter, fra små molekyler til patogener i vand, fødevarer samt i biologisk krigsførelse.

I fremtidens landbrug bliver dyrkning af afgrøder koncentreret i storlandbrug. Dette betyder, at landmanden har brug for detaljeret viden om hvilke dele af markerne der er inficeret med plantepatogener. Manuel markovervågning er dog dyrt og tidskrævende. Biosensorer, der er i stand til at detektere specifikke plantepatogener i sygdomsramte områder, vil give landmanden mulighed for at foretage pletsprøjtning på de inficerede områder, hvilket vil føre til en reduktion i brugen af sprøjtemidler. Det ideelle scenarie i præsitionslandbrug er, at man har billige og robuste biosensorer, der kan styres af personale med begrænset eller ingen træning i plantepatologi og som kan detektere analytten i jord- og vandprøver.

I denne afhandling fokuseres på at udvikle immunologiske sensorer til detektion af to modelorganismer, hhv *Puccinia striiformis* f.sp. *tritici*, der forårsager hvede gulrust og *Phytophthora infestans*, som er skyld i kartoffelskimmel.

Da der ikke fandtes noget antistof rettet mod urediniosporer fra *Puccinia striiformis*, blev monoklonale antistoffer fra mus (mAbs) produceret og karakteriseret. IgM-type mAbs fra ni hybridoma cellelinier blev testet for krydsreaktivitet mod repræsentanter fra almindelige genera. To specifikke mAbs blev udvalgt til nærmere analyse og brugt til at udvikle et kompetitivt ELISA (ved brug af mAb4) og et subtraktivt inhibitions ELISA (ved brug af mAb8). Det subtraktive inhibitions ELISA var mest følsomt med en detektionsgrænse på 1.5×10^5 urediniosporer/ml. Assayet er opbygget af en præinkubering af urediniosporer og mAb8, fjernelse af urediniospore-bundet mAb8 ved centrifugering og kvantificering af det resterende

mAb8 med et kanin anti-mus IgM antistof. Faldet i mAb8 koncentration kan herefter relateres til mængden af urediniosporer i prøven. Metodens stabilitet blev undersøgt ved at teste andre rustsvampe for krydsreaktivitet. Der blev fundet krydsreaktivitet mod *Puccinia recondita* og *Puccinia hordei*, hvilket indikerer at det ~ 39 kDa mAb8-antigen kunne være en konserveret strukturel komponent i overfladen af *Puccinia* arter.

Det subtraktive inhibitions assay blev videreudviklet til detektion uden mærkning ved at bruge en Surface Plasmon Resonance sensor. Kanin anti-mus antistoffet blev immobiliseret på en sensoroverflade og brugt til at kvantificere mAb8. Optimale regenereringsbetingelser blev identificeret og 20 mM HCl fungerede som en effektiv regenereringsopløsning. Surface Plasmon Resonance sensoren havde en detektionsgrænse på 3.1 x 10^5 urediniosporer/ml. Ved brug af sensoren blev *P. striiformis* endvidere detekteret i en opløsning af rustsvampen *Melampsora euphorbia*, hvilket indikerer en god specificitet.

En Surface Plasmon Resonance immunosensor blev endvidere udviklet til detektion af *P. infestans* sporangier. Et eksisterende *Phytophthora* genera mAb (phyt/G1470) var specifikt i tests mod sporer fra ascomycetes, deuteromycetes og basidiomycetes i et subtraktivt inhibitions ELISA. Det subtraktive inhibitions assay blev overført til en Surface Plasmon Resonance sensor og optimale assay og regenereringsbetingelser blev identificeret. Ud fra kalibreringskurven blev en detektionsgrænse på 2.2 x 10^6 sporangier/ml fundet. Assayet har en analysetid på 75 minutter, hvilket er bedre end eksisterende antistof- og nukleotidbaserede assays til *P. infestans* detektion.

Resultaterne i denne afhandling beskriver for første gang brugen af Surface Plasmon Resonance immunosensorer til detektion af plantepatogener og er det første skridt på vejen mod implementeringen af immunosensorer til markovervågning.

ABBREVIATIONS

CDR	complimentary determining region
EDC	N'-(3-dimethylaminopropyl) carbodiimide hydrochloride
ELISA	enzyme-linked immunosorbent assay
Fab	fragment, antigen binding
Fc	fragment, crystallizable
FITC	fluorescein isothiocyanate
IFA	immunofluorescence assay
Ig	Immunoglobulin
ITS	internal transcribed spacers
LFIA	lateral-Flow immunoassay
mAb	monoclonal antibody
MIA	microsphere immunoassay
NHS	N-hydroxysuccinimide
pAb	polyclonal antibody
PCR	polymerase chain reaction
PEG	polyethylene glycol
QD	quantum dots
rAb	recombinant antibody
RU	response units
scFv	single chain variable fragment
SPR	surface plasmon resonance
TIR	total internal reflection

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Peter Skottrup Hansen

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1. INTRODUCTION

1.1 Immunoglobulins, a universal tool

1.1.1 Antibody structure

Antibodies or Immunoglobulins are produced by plasma B-lymphocytes and function as a part of the immune system in mammals in the battle against disease. The introduction of foreign antigens, such as pathogens and toxins, into an organism starts a complex immune response, in which antibodies play an important role. Antibodies recognise antigens in a lock-and-key type of mode, which labels the foreign molecule or pathogen for destruction and removal. In modern diagnostics and basic research, antibodies have become key molecules as it is possible to develop specific antibodies against almost any component, from small drug molecules to intact cells.

The main architecture of most immunoglobulins consist of two heavy chains (50-70 kDa) and two light chains (~ 30 kDa), which have constant domains (IgG and IgD have C_H1, C_H2, C_H3 and C_L, whereas IgE and IgM have an extra heavy domain, C_H4) and variable domains (V_H and V_L). The special structure of the domains is termed the immunoglobulin fold and is composed of two β-sheets stacking against each other by the interaction of hydrophobic amino acids on each sheet (figure 1.1 A). Heavy chains are bound together by disulfide bridges and non-covalent bonds, such as salt-bridges, hydrophobic bonds and hydrogen bonds. In addition one light chain is attached to each heavy chain by the same kind of covalent and non-covalent bonds (figure 1.1 B). Immunoglobulins can be divided into five different classes (IgG, IgM, IgA, IgE and IgD), based on differences in the amino acid sequences in the constant region of the heavy chains. Within the different immunoglobulin classes subtypes can occur (for example mouse IgG1, IgG2a, IgG2b and IgG3). IgM is the first immunoglobulin class produced in response to antigen, but class switching later results in expression of IgG, IgA and IgE with the same antigenic specificity. All immunoglobulins within a given class have very similar heavy chain constant regions. IgG, IgD and IgE have a general Y-shaped structure, whereas IgM has a pentameric structure (figure 1.1 B). IgA is a dimer of two Y-shaped structures linked together (not shown in figure 1.1). The active regions of immunoglobulins are the two antigen-binding

fragments (Fab) and the constant region (Fc). Both heavy and light chains contribute to the Fab-regions, while the Fc region consists of the heavy chains only (C_H2 and C_H3). The Fc region is attached to Fabs via the linker region (figure 1.1). Antibodies are bivalent and the antigen binding part (i.e. complimentary determining regions, CDR) of the molecule is located on the tip of the two Fab domains, whereas the stem Fc domain mediate effector functions (*27*). In addition to intact immunoglobulins, antibody fragments (Fab, Fab_2, scFv) with antigen binding ability can be generated by proteolysis or by antibody engineering (figure 1.1 B).

The following sections will focus on classical antibody production and modern recombinant antibody development. Furthermore, principal aspects of antibody production against fungal antigens will be reviewed.

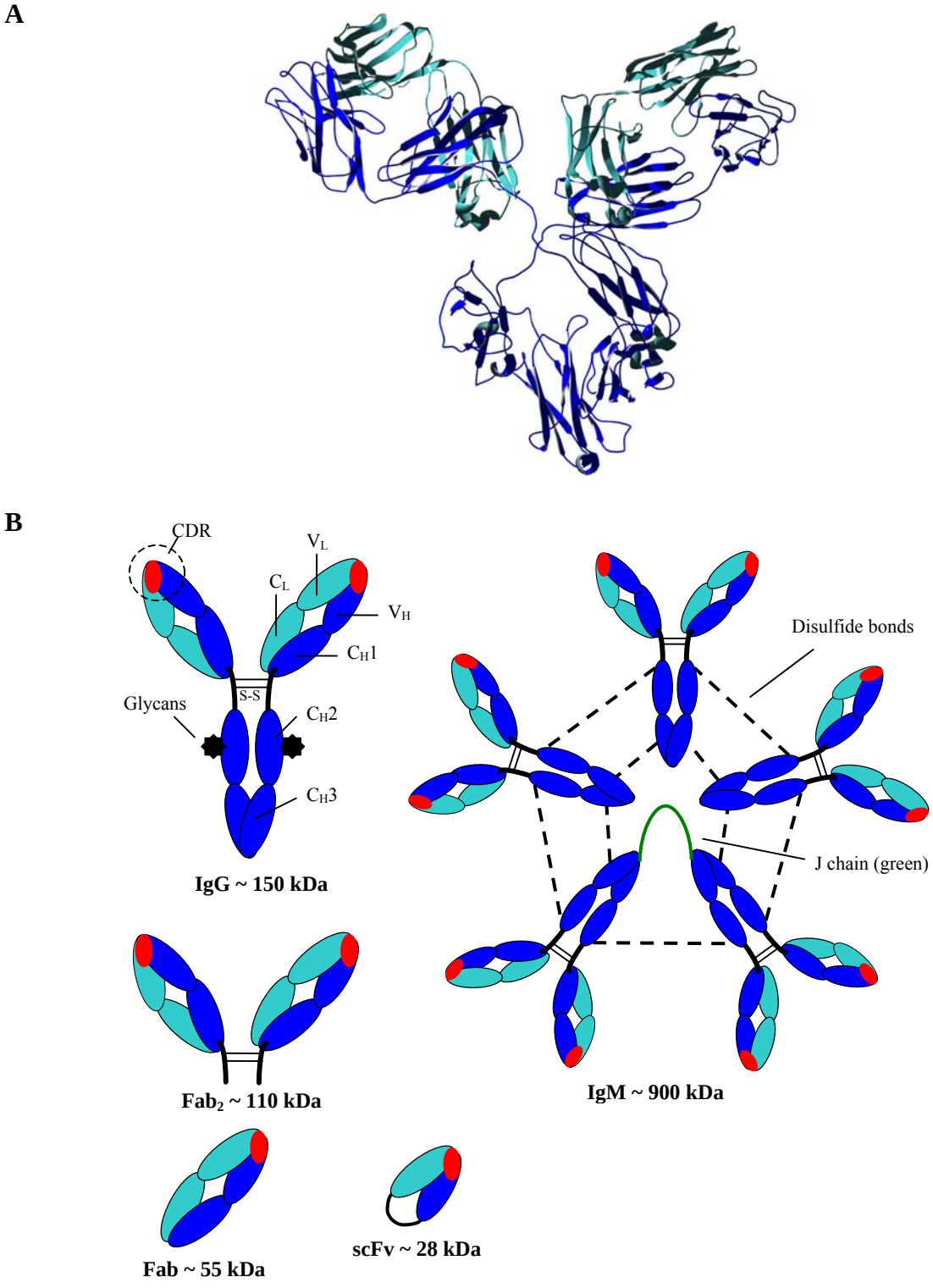

Figure 1.1: Overview of IgG and IgM structure. **Panel A** shows the three-dimensional structure of an IgG1 molecule determined by X-ray crystallography. The heavy chains are shown in dark-blue and the light chains are seen as light-blue. The coordinates from PDB 1IGY (*34*) was used to generate the structural representation and colour coding using swiss Pdb-viewer and quality enhanced using POV-Ray version 3.6. **Panel B** shows a simplified model of IgG and IgM structure (for simplicity the extra C_H4-domain is not shown in the IgM figure) using the same colour coding as panel A. The CDR-regions are seen as red dots. Heavy and light chains are linked through disulfide bonds (S-S) as well as non-covalent bonds (not shown). Glycans are seen attached to the C_H2 domains. Seen are also the most common antibody fragments.

1.1.2 Polyclonal and monoclonal antibodies

Antibodies are made by immunising a suitable mammal with the antigen. The host immune system will react with the antigen and B-lymphocytes will produce antibodies against the target. Several different B-lymphocyte clones produce antibodies, which are therefore termed polyclonal antibody (pAb). pAbs can easily be purified from the blood of the mammal by chromatographic techniques. A pAb raised against an antigen bind different epitopes on the target, which gives an increased risk that pAbs cross-react with biomolecules containing similar epitopes. Furthermore, the supply of pAbs is limited as the mammal is eventually killed. To circumvent these limitations Kohler and Milstein produced monoclonal antibody (mAb) producing cells in 1975 (*51*). This Nobel Prize winning work (1984, Physiology and medicine) revolutionised antibody production and today it forms the basis of many diagnostic applications, disease therapy and basic research (*27*). mAbs are antibodies of a single idiotype produced by immortalised B-lymphocytes recognising a single epitope on the antigen. Normal B-lymphocytes are fully differentiated and cannot be maintained in culture. Kohler and Milstein fused antibody producing B-lymphocytes with myeloma cells, thereby creating immortal antibody producing cell lines (hybridoma cells). As in pAb production, a suitable animal is immunised (usually mice or rats) with the antigen and after a sufficient serum antibody titer is detected, the animal is sacrificed and the splenocytes recovered. The splenocytes are fused with myeloma cells using polyethylene glycol (PEG) (*32*). The fusion is random and fused hybridoma cells must be selected and isolated from unfused B-lymphocytes and myeloma cells. The selection process is performed in medium which only allows for hybridoma survival. The cells are cultured in hypoxanthine-aminopterin-thymidine (HAT) medium. Aminopterin (A) blocks the *de novo* biosynthesis of purines and pyrimidines essential for DNA synthesis. When this pathway is blocked, cells use the salvage pathway utilising Hypoxanthine (H) and Thymidine (T), and this requires the activity of the enzymes Thymidine Kinase (TK) and Hypoxanthine-Guanine Phosphoribosyl Transferase (HGPRT). The myelomas selected for the fusion lack the HGPRT, so that unfused myeloma cells and myeloma cells fused to other myeloma cells cannot proliferate in HAT medium. The unfused splenocytes do possess HGPRT but have a limited lifetime and the culture will die within two weeks. The hybridoma cells grow effectively in the HAT media. Many different hybridomas are developed during the fusion and every cell type produces a specific antibody

towards a wide range of antigens and not only the antigen used. To identify the correct hybridomas, cells are distributed in 96-wells plates and hybridoma supernatant is used in Enzyme-linked immunosorbent assay (ELISA) to detect the positive wells for subsequent cloning by limiting dilution (*54*). The method essentially consists of diluting the cells and growing them at very low densities, often in the presence of feeder cells, which supply growth factors, see figure 1.2 for an overview of mAb production.

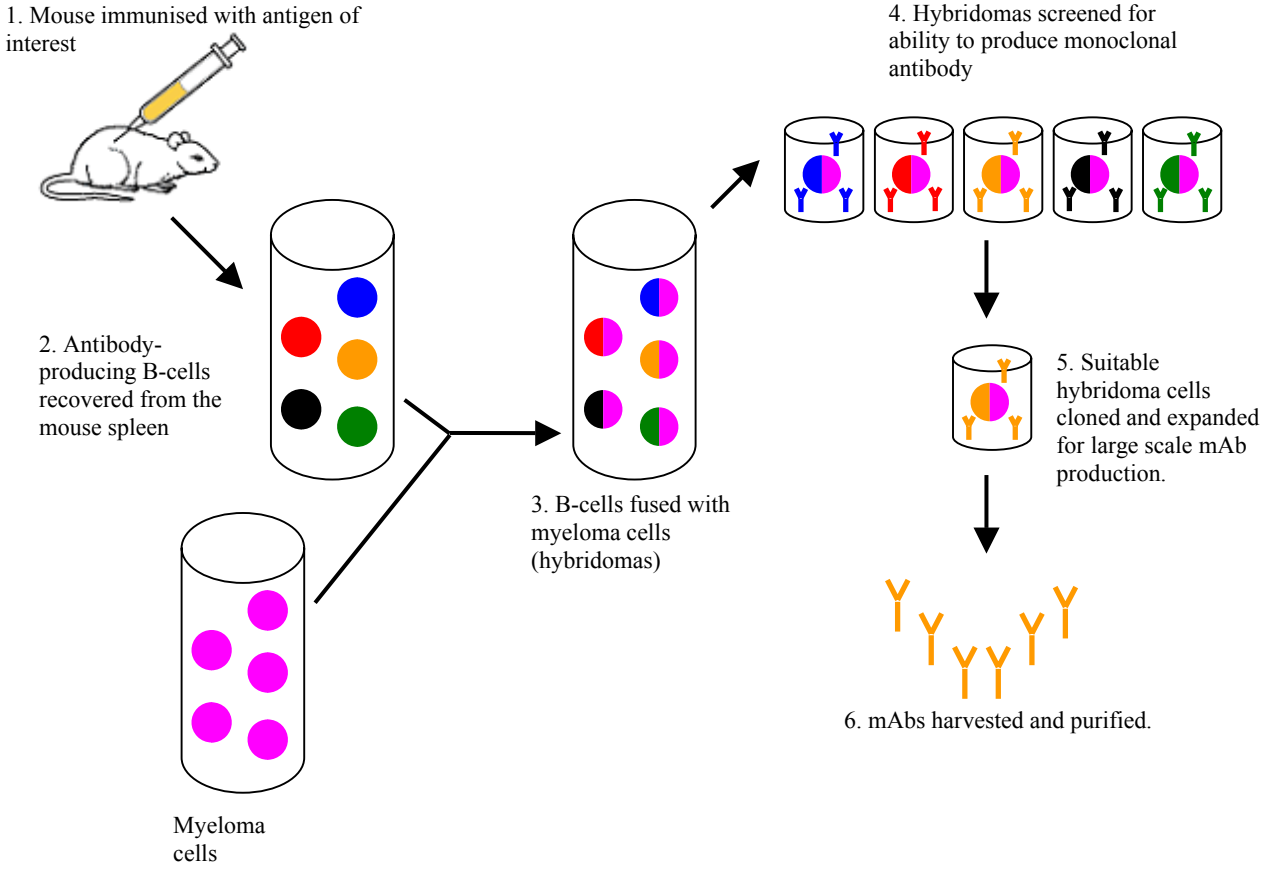

Figure 1.2: Overview of mAb production, see text for detailed description.

After screening, positive wells containing only one visible clone are selected for another round of cloning. After at least three rounds of cloning, the cell line is per definition monoclonal. Cloning continues until a stable hybridoma is achieved, that do not loose the mAb-producing ability (i.e. cells seeded out in a whole plate give positive signal in all wells). The cloned and stable hybridomas can then be expanded for large-

scale mAb production. Hybridomas can furthermore be frozen for storage and in this way an unlimited supply of mAb's can be maintained.

1.1.3 Recombinant antibodies

Recent advances in molecular biology have transformed antibody production. The development of recombinant antibody (rAb) technology has been stimulated by the failures of several attempts to make therapeutic mAbs against various diseases (*72*). The principal problem of therapeutic mAbs lies in the inherent antigenic properties of mammal mAbs as they are introduced in humans. The human immune response will react towards the mAbs, which tends to reduce the effectiveness of the drug. As a result of these initial failures, researchers have sought to "humanise" mAbs to reduce their immunogenisity. Furthermore, these "designer" antibodies can be made smaller, have increased affinity or be conjugated to therapeutic/diagnostic ligands, such as radiolabels for imaging or toxins for cell-targeted therapy (*39;72;78*). Basically the "humanisation" of murine or rat mAbs is performed by molecular engineering of the mAbs to replace native structures with their human counterparts by grafting murine Fab or CDR's onto a human scaffold. The diagnostics field have also benefited immensely from the emergence of these technologies, as antibody fragments can be produced quickly and cheaply without the need for specialised equipment (*68*).

Phage display technology is a powerful technique for rAb production. The technique was first introduced by the pioneering work of Smith in 1985, in which the construction of a phage virus carrying coat fusion protein was described (*77*). Later specific phage virus with epitopes fused to coat proteins was isolated from a mixture of phages; phage display was born (*66*). The basics of antibody phage display are a diverse mixture of filamentous phage (phage library) with foreign antibody encoding sequences spliced into phage coat proteins (pVIII, pIII or pVI). The peptide encoded by the foreign DNA is displayed on the surface of the virion, fused to the coat protein. Each phage clone displays a single peptide and libraries can consist of billions of different clones. The viral carrier is infective to bacteria and whole libraries or single clones can be propagated indefinitely. In development of phage antibodies the displayed peptide is a domain of an antibody molecule, including the CDR region. Phage antibody libraries are produced by amplification of

highly conserved terminal regions of V_H and V_L genes by Polymerase Chain Reaction (PCR) using specific primers. The amplified PCR products are then assembled in vectors thereby creating repertoires of antibody fragments (*1*).

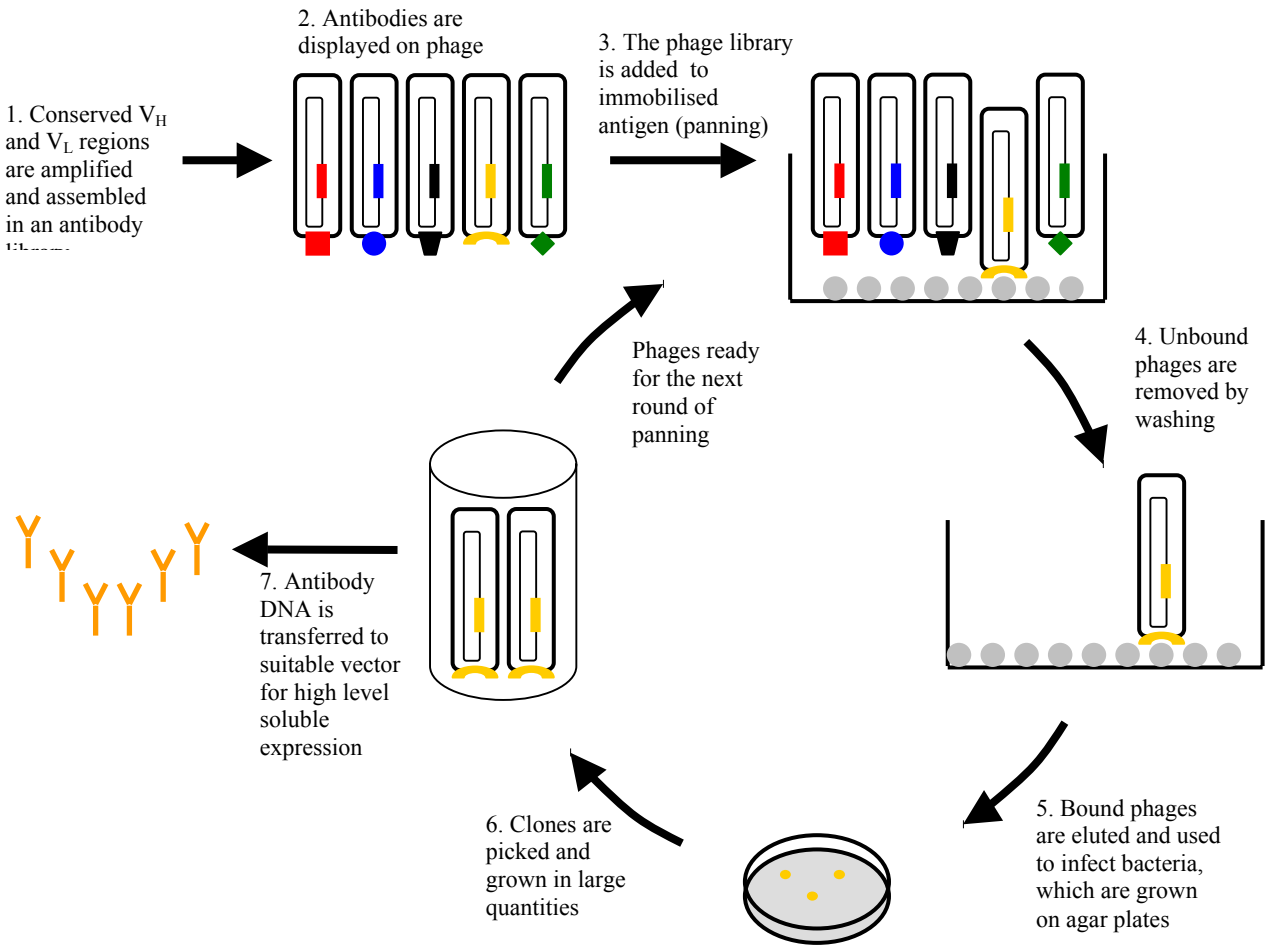

Figure 1.3: Overview of antibody selection by phage display. Individual antibody-encoding DNA is seen in different colours integrated within the phage genome. After translation the antibodies are displayed on the phage surface as a fusion product to pIII. Phages are added to antigen coated microtiter wells for selection of binders. Non-binders are washed away. Binders are eluted (typically by low pH) and used to infect bacteria followed by plating out on agar plates. Clones are picked for amplification and subjected to the mext round of panning See text for more details.

The phage display allows for affinity selection by antigen *in vitro* (termed panning), which is analogous to antibody selection in natural immunity (figure 1.3) (*27*). Panning is the incubation of phage in antigen coated microtiter wells, where binding clones are captured, and non-binders are washed away. Bound phages are detected using enzyme-conjugated anti-phage antibodies. The binding clones are eluted and used to reinfect bacteria, for proliferation and the next round of panning. After sufficient rounds of panning (usually 2-3 rounds), isolated phage clones are propagated in bacteria to characterise the displayed antibodies. The antibody genes can be transferred to high-yield expression systems for soluble expression. The source of immunoglobulin genes in phage display libraries may be isolated from B-cells of immunised mammals (animal or human) or from naive B-cells (from non-immunised animals). The advantage of using naive libraries is that one phage antibody library can support the development of antibodies against a wide range of antigens. Different types of antibody fragment can be generated by phage display and the most popular are Fab-fragments and scFvs (heavy and light chain variable regions linked by a short peptide) (figure 1.1). rAbs are becoming increasingly popular primarily due to the relative ease of production, low cost (compared to hybridoma mAbs), similar affinity as conventional mAbs, the possibility of increasing binding affinities by engineering and the fact that animals are not required for production (*1*).

1.2 Fungal antigens and considerations in antibody production

1.2.1 Fungal antigenic determinants

The surface of fungal pathogen cell walls may act as immunogens. The Cell wall of fungi contains many different protein and carbohydrate components, which can elicit an immune response. mAbs have been found to target carbohydrate epitopes in fungal species and especially mannosyl and glycosyl residues are widely targeted (*87*). Antigenic surface proteins have also been targeted by many mAbs. For some antibodies the target have been found to be of a polypeptide nature, but glycoprotein targets have also been found, in which both polypeptide and glycans contribute to the epitope (*22;31;60;87*).

The strategy for antibody development is dependent on the application of the antibody. This means that the antigen used for antibody production should be a as close as possible to what one wants to detect in the final assay (*22;31;60;87*). It is recognised that pAbs often do not have the required specificity in fungal immuno assays, which is likely due to the presence of shared immunodominant glycan structures on fungal surfaces (*22;31;60;87*). Therefore, mAbs have been extensively used to discriminate between fungal species. Although there is great variability in antigen preparation in the literature, some general trends can be observed and three main antigen types are used for mAb production. (i) Homogenised mycelium is often used as antigen (*7;13;21;26;38;61;63*). The mycelium components are extracted in liquid nitrogen and ground to a fine powder using a mortar and pestle. Alternatively the mycelium is freze-dried and then pulverised (*7;38*). The powder is solubilised and typically mixed with Freunds adjuvants for immunisation. The produced mAbs are often suitable for pathogen detection in homogenised leaf material, but mAbs have been produced that react with mycelium but not spores of the same species (*87*). (ii) Intact spores or spore fragments have also been used as antigen in mAb production (*41;52;73;75;76;89*). Due to different growth procedures for individual fungi, spores are prepared in different ways, but the most common approach is to extract spores from agar-plates by buffer-wash (*73;75;76*). Spore-containing buffer is then used for immunisation, in conjunction with adjuvant. Using spore fragments, intracellular antigens (typically enzymes or ribosomal proteins) will be released and consequently mAbs can be raised against structures that are not present on intact pathogens. By screening the hybridomas against intact pathogen the risk of obtaining these mAbs can be minimised (*22;31;60;87*). (iii) By washing the surface of spores or mycelium with buffer surface components can be extracted (*11;20;82*). These are often (glyco-)proteins, (phyto-)toxins and extracellular polysaccharides, either secreted by the fungi or ”true” surface-molecules loosely-associated with the pathogen surface (*87*).

1.2.2 Pathogen processing by the immunesystem

As particulate matter, such as fungal spores, is injected into mammals for antibody production, the host immunesystem must degrade the particles into smaller molecules, which is most likely performed by the complement system. The complement system lyses fungi upon activation by cell-surface constituents that are

foreign to the host, such as cell wall components of spores (*27*). Degraded fungal antigens crosslink membrane-bound immunoglobulin (mIg) on mature B-cells, which leads to B-cell internalisation of the antigen-antibody complex. The antigen is further degraded within compartments of the endocytic processing pathway of B-cells by enzymes. Fungal-derived peptides associate with class II Major Histocompatibility Complex (class II MHC) and are displayed on the cell surface. The knowledge of surface presentation of processed non-peptide antigens is limited, but current belief is that these molecules are presented by members of the CD1 family of nonclassical class I molecules (*27*). The surface displayed class II MHC-antigen complex is recognised by the T-cell receptor of helper cells (T_H cells), which release cytokines that drives the differentiation of the B-cell into antibody-secreting plasma cells and memory cells. Activation of B-cells can also occur independent of T_H cell interaction in which the binding of special antigens to B-cell mIg drives the differentiation. However the differentiation is still dependent upon the T_H-cell cytokine production. Furthermore, the response to these antigens (e.g. bacterial lipopolysaccharide) is generally weaker, no memory cells are formed and IgM is the main antibody type generated, illustrating a low degree of class switching (*27*).

1.2.3 Limitations of mAbs and a role for rAbs

Upon lysis of pathogens, intracellular antigens are released. This means that these antigens can potentially encounter a mature B-cell and trigger production of a specific antibody against an intracellular antigen. After fusion of B-cells and myeloma cells in mAb production, hybridomas secreting mAb against intracellular antigens can therefore be produced. Consequently, care should be taken in the choice of antigen in the mAb screening process (e.g. using intact pathogens) to limit the selection of mAbs targeting intracellular antigens. An alternative to traditional mAb production is rAb production. As mentioned earlier, rAbs targets a specific epitope like mAbs but are less costly and requires less specialised equipment for their production. However, their main advantage is that the panning process occurs *in vitro*, meaning that the rAbs can be selected against unmodified antigens. This makes rAbs particular suitable for targeting native surface structures on pathogen surfaces and several researchers have utilised the potential of rAbs as specific pathogen reagents (*8;14;28;68;88*).

1.3 Established and emerging immunoassays for pathogen detection

1.3.1 Enzyme-linked immunosorbent assays

The most popular application for immunological detection is the Enzyme-Linked Immunosorbent Assay (ELISA). The simplest system is Plate Trapped Antigen ELISA (PTA-ELISA), in which surface-bound antigen is probed with antibodies carriyng covalently attached enzyme molecules. Antibody binding immobilises enzyme in the vicinity of the bound antigen, allowing detection of the antigen after substrate incubation (see figure 1.4A). Variations include competitive ELISAs in which surface-bound antigen and antigen in solution competes for antibody binding. In this system, comparison of signal with signals from known antigen standards allows very accurate quantification. In Double Antibody Sandwich ELISA (DAS-ELISA), surface-bound antibody is used to capture the antigen, followed by detection using a second enzyme-labelled antibody. Sandwich ELISAs are extremely specific as the antigen must react with 2 antibodies to be detected. Typically, antibodies are conjugated to alkaline-phosphatase or peroxidases and the signal observed as a colour development following substrate incubation. ELISAs are run in 96 well plates and scanned by automated devices. Several commercial DAS-ELISA's exist for plant pathogen detection and these are mostly used for detection of pathogens in homogenised plant tissue.

1.3.2 Immunofluorescense microscopy assays

In direct Immunofluorescence Assays (IFA), the pathogen sample is fixed onto a microscope slide, and analysed with a drop of pathogen specific antibody labelled with a suitable fluorochrome (f.x. fluorescein isothiocyanate, FITC). Unbound FITC-conjugate is rinsed off, and the slide is examined under an epifluorescent microscope. If antibody has bound, the sample will display a green fluorescense signal, not present in control samples. Indirect IFA's can also be performed, in which pathogen-specific antibody is detected by FITC-labelled anti-species immunoglobulin (*27*). IFA methods are laboratory-based and require an epifluorescent microscope equipped with appropriate excitation and emission filters. Also, relatively few samples can be examined per day, and the analysis of results is subjective and requires experienced personnel.

1.3.3 Lateral-flow immunoassays

Lateral-Flow Immunoassays (LFIA) is becoming increasingly popular as on-site diagnostic tools for plant pathogen detection. The assays consist of an immunoreaction and a chromatography step (often termed immunochromatography) and the power of the technique lies in the speed of analysis. The technology has been available for many years in clinical diagnostics. The most popular and well known is the home pregnancy test, which detects the glycoprotein hormone, human Chorionic Gonadotrophin (hCG) in urine of pregnant women. The assays involve the unidirectional flow of particles (e.g. latex, silica, carbon) coated with analyte-specific antibody along a nitrocellulose membrane. A basic sandwich type LFIA exemplified by viral detection is seen in figure 1.4B. Applied samples are transferred along the membrane by capillary flow, which allows for good sample separation as the reactant site is different from the application site. The antibody-coated particles are present in the application site, where the particles 'capture' the antigen as they are applied to the strip. As the antigen-particle complex migrates in the membrane, the complex is selectively bound by an immobilised antigen-specific antibody, thereby generating a solid line visible to the eye. Particles that did not bind antigen continue to migrate up the membrane, where they are bound by an anti-species antibody. For a positive result both the test line and the control line must appear (*17*). In addition to the sandwich type assay described here, competitive and inhibition type assays exist. LFIA's are user friendly, relatively inexpensive, and suited for on-site testing by minimally trained personnel. However, LFIA's have major limitations as only qualitative results are obtained (i.e., it gives a "yes" or "no" answer).

1.3.4 Dipstick assays

Different Dipstisk assays types exist. One of the widely used consists of a dipstick (typically nitrocellulose) coated with pathogen specific antibody. The dipstick is emerged into the sample and if present, the pathogen is specifically captured by the antibody. Following extensive washing of the dipstick, a second pathogen-specific enzyme-conjugate antibody is added. After incubation in enzyme substrate, a colour development is indicative of pathogen contamination. Another format consists of a blank dipstick which is emerged into the sample. Pathogens will bind the dipstick and enzyme-conjugated pathogen-specific antibody is added. Again a colour development after the addition of enzyme substrate, suggests the presence of the pathogen in

question (*19*). As with LFIA analysis, these methods are fast and can be operated by non-scientifically personnel, but quantitatively data are hard to obtain.

1.3.5 Multiplex immunoassays

Promising new assay types, termed multiplex immunoassays have been developed in recent years Figure 1.4C). Multiplexing is defined as the detection of several pathogens simultaneously and is usually performed on the Luminex system. The technology is based on the use of beads (microspheres of 5.6 microns), which are internally stained with two fluorochromes. A hundred different bead sets are available, with unique compositions of red and infrared fluorochromes. This makes detection of up to 100 different antigens possible. Both antibodies and nucleic probes can be covalently linked to the activated microspheres. In a Microsphere Immunoassay (MIA), samples are incubated with antibody coated beads. The pathogen/antigen are caught by the bead-antibody whereafter secondary antibodies, conjugated with a reporter dye, are added, resulting in a sandwich type of assay. The samples are applied to a Luminex analyser, where one laser excites the internal dyes to identify each microsphere particle, and another laser excites the reporter dye on the secondary antibodies captured. This gives detailed data of the presence of pathogen. Several different beads (with different antibodies attached) can be added to one sample, which can thereby be scanned for the presence of many different pathogens (*86*). The technique has successfully been used for multiplex detection of three viral plant pathogens and four bacterial plant pathogens (*85*).

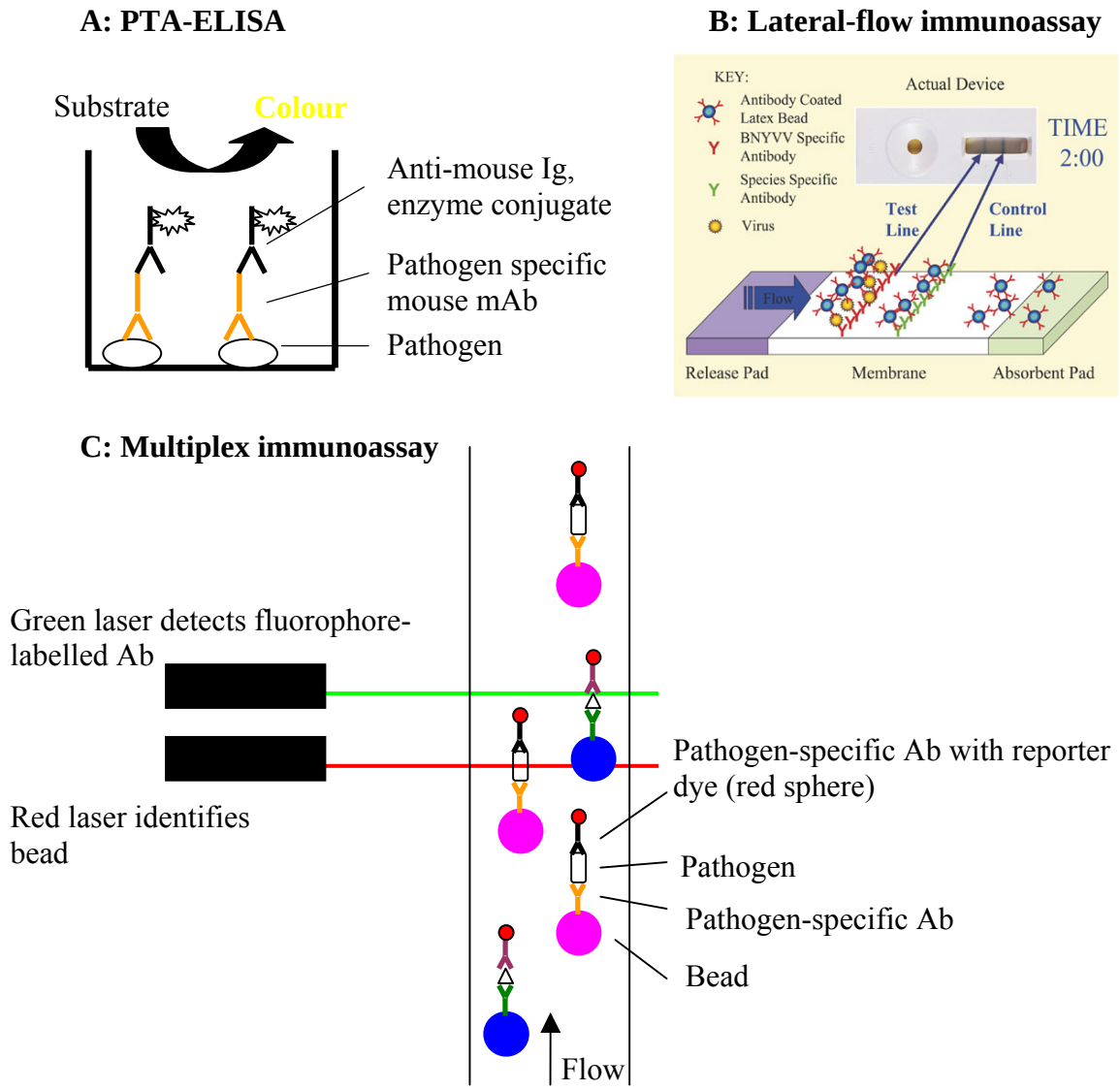

Figure 1.4: Overview of established and emerging immunoassays. A detailed description of each assay type is found in the text. Panel B is from pocketdiagnostic.com.

1.3.6 Quantum dot based assays

Quantum Dots (QDs) are inorganic semiconductor crystals that fluoresce at sharp and distinct wavelengths depending on their size. QD are nanometer scale (~ 10-20 nm, approximately protein-sized) and usually consist of a CdSe or CdTe core and ZnS shell. QD's have broad absorbance ranges, meaning that a single excitation wavelength can excite QDs of multiple emission maxima (*59*). For this reason QD holds

promising applications as components of multiplex assays as different size QDs can be functionalised with different biomolecules. In addition to the multiplexing capability another advantage of QD's is their ability to retain high fluorescence for hours in comparison to typical organic dyes, which bleaches within seconds (*59*). QDs can be used in biological assays due to coatings with organic material that make QDs water soluble. The coating is designed so that biomolecules can be conjugated to the surface and antibody-QD-conjugates holds great promise as tools in immunoassays (*59*). In a typical experimental setup, different QDs coated with different antibodies binds their respective antigens. The binding is visualised by simultaneously exciting the QDs, leading to emission of two different colours. A limited number of studies into the use of antibody-QDs for cell detection have been reported. Hahn and co-workers, detected *Escherichia coli* using antibody-QD conjugates and found a 2-order of magnitude increased sensitivity compared to standard fluorescense assays using a common dye (*29*). In recent work two different antibody-coated magnetic beads were used to concentrate *Escherichia coli* and *Salmonella Typhimurium* from samples, followed by visualisation of the bead-cell-QD complexes by fluorescence microscopy (*90*). Although these studies have shown promising results, it is recognised that a lot of developmental work in surface conjugation and assay reproducibility has to be adressed (*59*).

1.4 Taking the lab into the field

1.4.1 Biosensors for detection of plant pathogens

All of the above described immunological methods for pathogen detection are dependent of labeled antibodies (i.e. with enzymes, flourescence tags or small particles). This means that these methods are primarily confined to laboratory environments with equipment that can detect the labels. A lot of effort has been put into the evaluation of biosensors for pathogen detection, which holds the potential to be implemented for on-site analysis. A biosensor is an analytical device incorporating a biological or biologically derived sensing element either integrated within or intimately associated with a physicochemical transducer (*84*). The aim of a biosensor is to produce either discrete or continuous digital electronic signal, which is proportional to the interaction of biomolecules. This means that the sensor essentially transforms a biological interaction into a digital signal, making detection of analytes label-free. Due to the specificity of the biological interaction a biosensor can be used in complex media such as blood, serum, urine, fermentation broths and food, often with minimum sample treatment (*2*). In addition to label-free detection, biosensors has the advantage of repeated analysis on the same surface, which can lower the cost of sensor use. The limitations of biosensors are the stability of the immobilised biological material and the uniformity and geometry of the ligand on the biosensor surface, which can affect reproducibility.

Different biosensor types exist; amperometric biosensors (detect changes in current), potentiometric biosensors (detect changes in potential), piezoelectric biosensors (detect changes in mass), calorimetric biosensors (detect heat changes) and optical biosensors (correlates changes in concentration, mass, or number of molecules to direct changes in characteristics of light). All these sensor types have been developed to meet the demand of faster and accurate detecting methods. Demands for sensitivity (detection of low analyte concentrations), specificity (detection of an analyte in complex matrices), speed and accuracy of bioelectronic measurements have driven the development of bioelectronic probe procedures mostly in food/water diagnostics and in biowarfare applications. Optical detection using Surface Plasmon Resonance

(SPR) biosensors has been increasingly popular due to its speed of detection, high specificity, high sensitivity and possibility of real-time analysis.

1.4.2 Surface Plasmon Resonance (SPR) sensors

The SPR biosensor technology allows the real-time detection and monitoring of biomolecular binding events. The Biacore® technology, which utilises the SPR phenomenon, was first introduced in 1990, by Pharmacia AB, Uppsala, Sweden and the technology is today widely used, due to its high sensitivity and speed of analysis. In a SPR experiment, one interacting molecule (ligand) is bound to the biosensor surface (the sensor chip) while the other (analyte) is delivered to the surface in a continuous flow through a microfluidic system (figure 1.5).

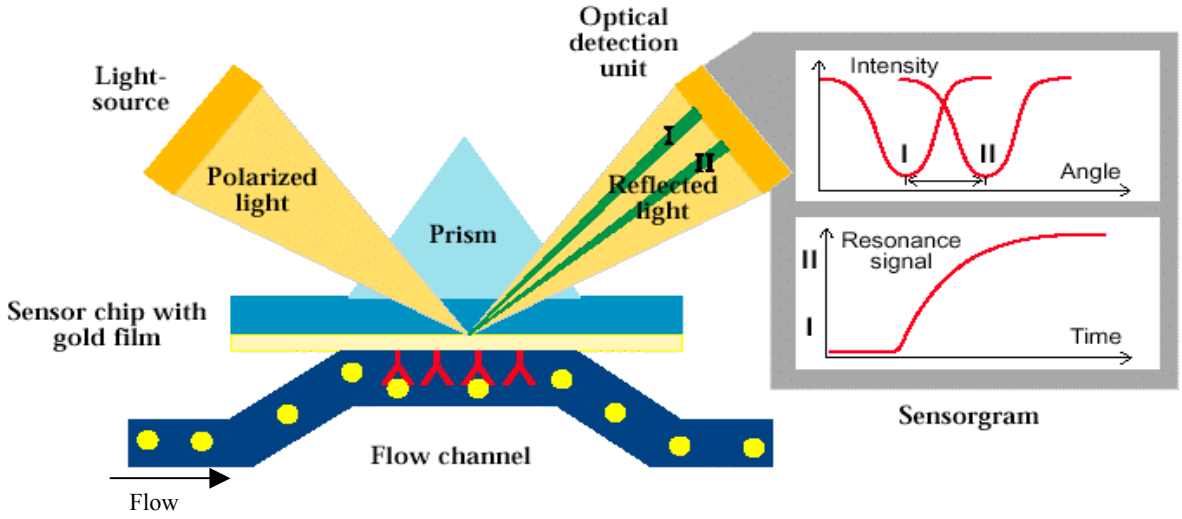

Figure 1.5: Overview of Biacore SPR technology. Ligand is seen as red Y-shapes and analyte is seen as yellow spheres. See text for more details. Figure is from biacore.com.

Binding of anlyte to the immobilised ligand is followed by SPR, leading to detection of mass concentrations at the sensor surface. The response is measured in Response Units (RU) and is proportional to the molecular mass on the surface. This means that for a molecule of a given mass the response is proportional to the number of molecules at the surface. 1000 RU typically corresponds to 1 ng of protein bound per mm^2, however the exact conversion from RU to protein amount depends on the sensor surface and the analyte

molecule. The data output of the technology is the sensorgram, which is a plot of response units against time. The technology requires no labelling of biomolecules and can provide quantitative information on kinetic parameters (association and dissociation rate constants) and measurements of active concentrations (*58*).

SPR technology is used to characterise molecular interactions involving small molecules, proteins, polysaccharides, lipids and nucleic acids, when studying epitope mapping, molecular assembly, ligand fishing and small molecule screening (*49;67*). Experiments are performed either with purified analytes or with analytes present in crude media (cell or bacterial lysates, tissue extracts, sera and biological fluids. Recently SPR sensors have been used for whole cell detection in various assay formats, which will be reviewed later.

1.4.3 SPR Detection principle

In SPR sensors, an interface between transparent media of different refractive index (glass and buffer) is used. When light is passing from the side with higher refractive index (glass) at a certain critical angle of incidence no light is refracted across the interface and Total Internal Reflection (TIR) is observed (*33*). At TIR, an electromagnetic field component, termed the evanescent wave, penetrates a short distance (approximately 300 nm in Biacore™ sensors, but depends on the SPR system) into the buffer. The interface between the glass and buffer is coated with a thin layer of gold and the photons in the incoming light react with the free electron cloud in the metal film at a specific angle, the SPR-angle. This causes a drop in the reflected light at the SPR-angle. The angle at which SPR occurs (the SPR-angle) is dependent on the refractive index of the buffer side within the evanescent wave range. Consequently, the binding of biomolecules to the sensor surface will cause a shift in the SPR-angle, which is directly proportional to the mass increase (*80*). Binding of biomolecules to the sensor surface can be followed in real-time by continuously monitoring the shift in the SPR-angle (the sensorgram), figure 1.5. The angular shifts are expressed in RU, where 1 RU corresponds to a shift of 0.0001° or approximately 1 picogram of protein (*43*).

1.4.4 SPR assay development

There are three major steps in a SPR assay, (i) ligand coupling to sensor surface, (ii) interaction analysis and (iii) regeneration of sensor surface. The following paragraph will review the crucial steps for successfull SPR biosensing, ligand coupling and surface regeneration.

1.4.5 Ligand coupling

Different strategies exist for the covalent coupling of ligands to the sensor surface. These include thiol-, aldehyde-, biotin-(strept)avidin- and amine-coupling. Here the focus will be on amine-coupling in relation to the Biacore® CM-series chips, which are the most widely used coupling chemistry and the one used in this thesis. The matrix on CM-series sensor chips is composed of linear carboxymethylated dextran, which is covalently attached to a gold surface and presents itself as a hydrogel under aquaeous conditions with a thickness of approximately 100 nm under physiological conditions (*57*). The matrix provides a hydrophilic 2-dimentional environment to biomolecules and ligands can be covalently linked to the carboxyl groups in the matrix. This means that there is a high degree of accessibility to the interacting molecular surfaces, which is in contrast to assays, where ligands are absorbed directly to a sensor metal surface and interaction with analytes can be sterically hindered by the surface or biomolecules in close vicinity (*57*). An overview of amine-coupling using N-hydroxysuccinimide (NHS) and N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is seen in figure 1.6.

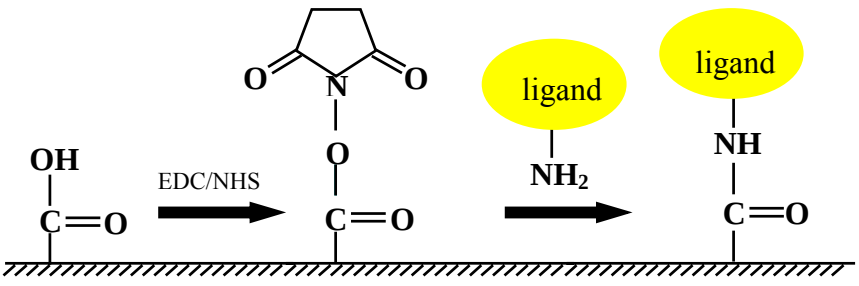

Figure 1.6: Immobilisation of protein to a dextran matrix using amine-coupling (*42*). See text for detailed description.

Briefly, the carboxymethyl groups in the matrix are modified into N-hydroxysuccinimide esters by EDC and NHS (for a detailed description of a typical immobilisation see chapter 4, paper 3). The esters produced are able to spontaneously react with amines and other nucleophilic groups on ligands to form covalent bonds (*42*). The ligand to be coupled must be as pure as possible and free from primary amines such as Tris, glycine and stabilising proteins (typically Bovine Serum Albumine (BSA) added to commercial antibody preparations). Secondary amines such as the preserving agent NaN_3 may also interfere in the coupling reaction. As the carboxymethylated matrix is negatively charged in the running buffer (pH 7.4), the ligand must have a positive charge so that it is not electrostatically repelled from the surface during the coupling reaction. By using coupling buffers with a pH below the isoelectric point of the ligand, the positive charge of the ligand can be achieved. The optimal pH for immobilisation (the highest charge attraction between ligand and surface) can quickly and easily be determined using a blank unactivated CM surface and injecting ligand in different pH buffers, a process called pH-scouting or preconcentration (figure 1.7A). The pH-buffer with the highest response is used for immobilisation. Unreacted esters are subsequently blocked with 1 M ethanolamine, which also releases loosely-bound antibody from the surface. A typical immobilisation sensorgram is seen in figure 1.7B exemplified by the immobilisation of rabbit anti-mouse IgG1 immunoglobulin.

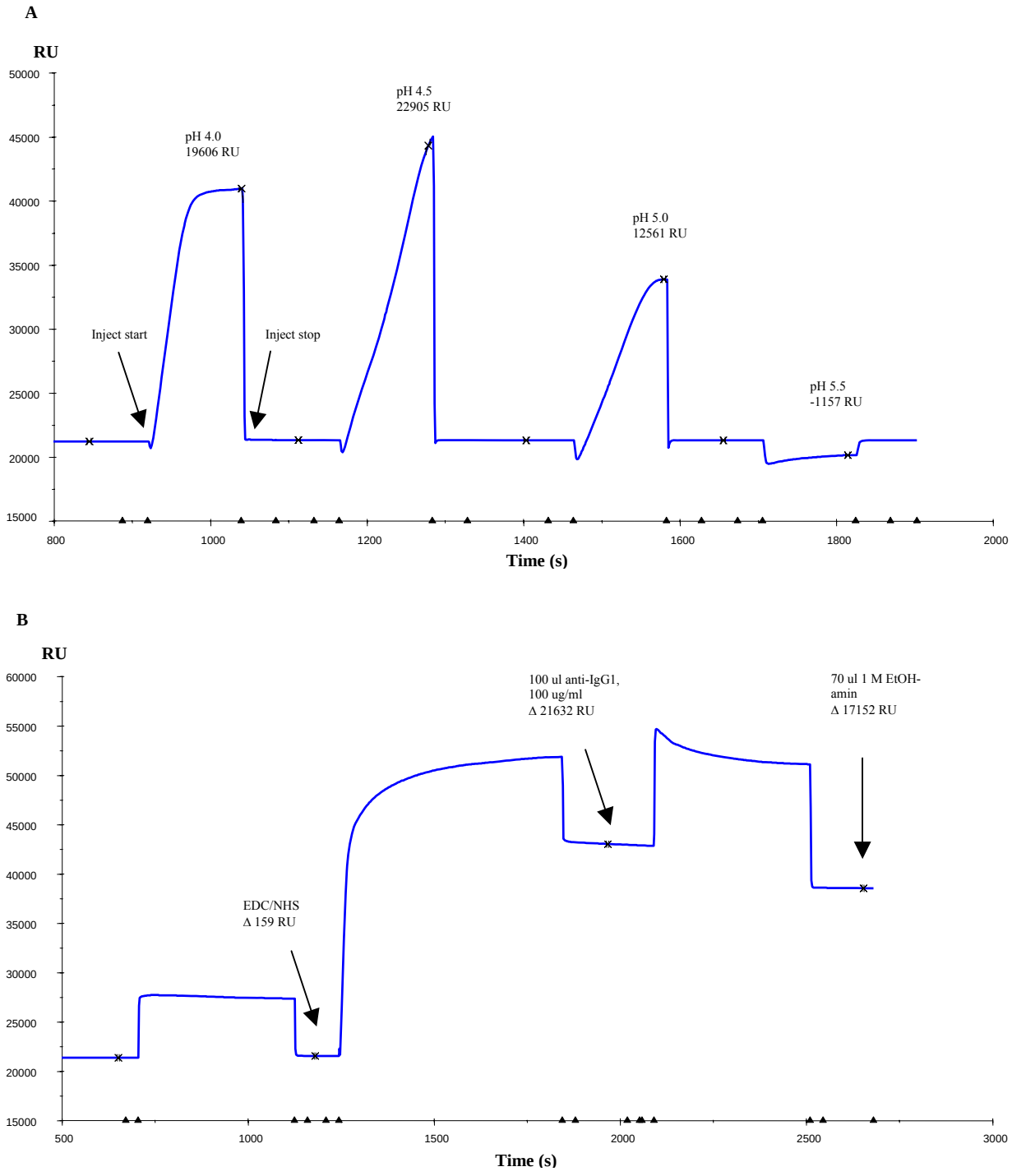

Figure 1.7: **Panel A** shows a typical pH-scouting experiment, exemplified by polyclonal rabbit-anti mouse IgG1. 10 µg/ml antibody solutions in 10 mM sodium-acetate buffers with different pHs are injected over a blank CM5 surface. The pH-buffer resulting in the strongest protein-attraction to the surface (seen by the highest peak, here pH 4.5) is used for subsequent immobilisation. By this technique it is also also possible to estimate the isoelectric point of ligands in a fast and precise manner. In this example the ligand is completely repelled from the CM5 surface at pH 5.5, suggesting that the isoelectric point of polyclonal rabbit-anti mouse IgG1is within the interval pH 5-5.5 **Panel B** shows a typical immobilisation of rabbit anti-mouse IgG1 at pH 4.5. First the CM5 surface is activated by EDC+NHS. As the surface is slightly changed due to the formation of esters a small increase in RU is observed (159). Next polyclonal rabbit-anti mouse IgG1 is injected over the surface leading to amine-coupling of the antibody to the surface (21632 RU). Unreacted surface groups are then blocked using 1 M ethanolamine, which also releases loosely-bound antibody from the surface, leading to a drop in RU. During this immobilisation 17152 RU was attached to the CM5 surface. For a detailed description of the immobilisation, please consult paper 3 in chapter 4.

1.4.6 Regeneration

With the latest developments in sensors for label-free detections of biomolecules, there is a need for repeated analysis on the same functionalised surface to reduce the price of assays. The goal is therefore to disrupt the non-covalent binding between the immobilised ligand and the analyte, without decreasing the activity of the ligand, thereby prolonging the lifetime of the surface. In most cases short pulses of 10-100 mM HCl or 10 mM Glycine-HCl pH 1.5-3.0 is sufficient to completely remove all non-covalently bound material from the surface, while maintaining the activity of the ligand. Low pH buffers are believed to partly unfold the proteins making them positively charged and the result is that binding sites no longer match and the molecules repel each other. However, sometimes other types of regeneration must be used if low pH buffers are not useful. These buffer types include either high pH, high salt, EDTA (ethylenediaminetetraacetic acid), chaotropic salts, ionic detergents, non-polar solvents and reducing agents (*3;4*). Good effects have also been demonstrated using cocktails of the different groups of regeneration solutions, thereby targeting several binding forces simultaneously (*3;4*).

Identifying optimal surface regeneration can be very difficult, time consuming and is sometimes never achieved. A good regeneration is however the most important parameter in immunosensor development for the assay stability and reproducibility. Figure 1.8 shows typical profiles of different patterns encountered with repeated analyte binding and surface regenerations (*3;4*). Ideally the baseline and response lines are straight (figure 1.8 A), but insufficient regeneration conditions can be used in assays provided that the binding response does not decrease over time (figure 1.8 B). Insufficient regeneration can however also result in a decrease in the response line over time as a result of accumulated analyte on the sensor surface blocking the binding sites (figure 1.8 C). Regeneration conditions can also be too harsh resulting in unwanted ligand inactivation (figure 1.8 D).

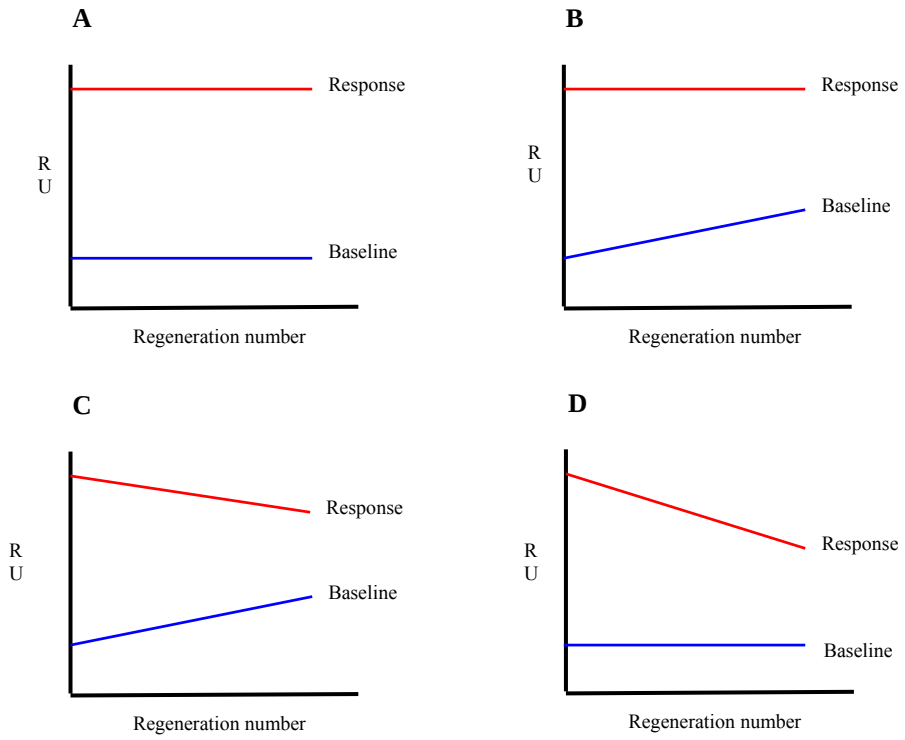

Figure 1.8: Exploring surface regeneration. **Panel A**: optimal regeneration is seen as both the baseline and response are stable over time after several regenerations. **Panel B**: insufficient regeneration is seen but a stable response is measured so the insufficient regeneration does not affect sensor performance. **Panel C**: Insufficient regeneration leads to accumulation of analyte at the sensor surface, which leads to a decreased response over time. **Panel D**: the surface reneration inactivates the immobilised ligand leading to a decreased response response over time. More details are found in the text.

1.4.7 Detection of microorganisms using SPR

Models explaining the principal aspects of multivalent binding to immobilised ligands are currently well established (*62*). However, models describing whole cell binding to fixed ligands are not widely available and no general consensus prevails. Unlike ELISA setups in which ligands binds pathogen under static conditions, in most SPR-configurations the pathogen has to be caught on the flight as the sample flows across the ligand surface. This means that several physical considerations has to be taken into account, when describing cell binding to immobilised ligands. The following paragraph will examine the available information of SPR whole cell detection in the context of Biacore® CM-series surfaces.

Several studies exist in which SPR has been used for whole cell detection, but until now the research has focused on technique refinement for reliable cell detection (*6*). All SPR studies on cell detection have been perfomed using bacterial cells. Different types of 'direct-capture' assay formats have been investigated but an apparent poor sensitivity of the SPR formats with respect to low concentrations of cells is a general trend (*6;36;65;69;70*). Several factors are believed to attribute to the limited sensitivity of SPR-based assays for whole pathogen detection, as illustrated in figure 1.9. The effective penetration depth of the evanescent field which arises under conditions of TIR is approximately 300 nm. Therefore, only refractive index changes occurring within this distance from the surface will cause a change in the generated SPR signal. Cells probably do not penetrate the CM5 chip dextran layer and therefore only a small portion of the cell which is in close contact with the sensor surface will contribute to refractive index changes (*55*). Furthermore, Biacore® instruments average the SPR angle over an area of approximately 0.25 mm^2 on the sensor surface. This means that the signal response can decrease as bacterial cells are large and may not evenly cover an area measured (*25*). Other major factors limiting sensitivity is the extreme 'bulk' and non-ideal viscoelastic properties, exhibited by cellular structures. Mass-transfer limitations play a role here and particulate fluid dynamics, suggest that fluid forces has to be overcome for particles to be captured on sensor surfaces (*79*). Once cells have found their target, a considerable high antibody-cell binding avidity must be able to withstand the effect of shear force created by the laminar flow (*6*).

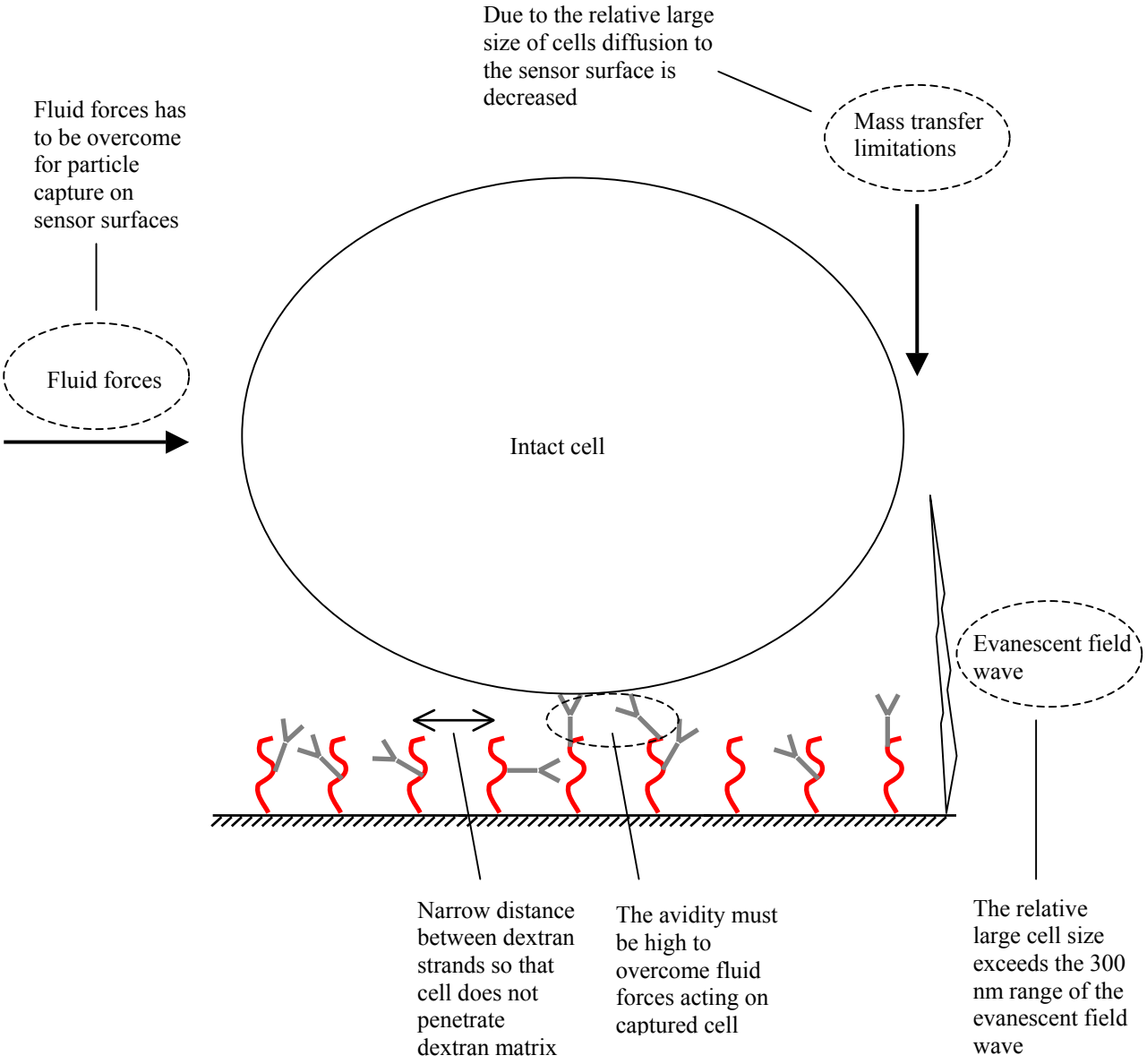

Figure 1.9: Overview of problems associated with whole cell capture on an antibody sensor in SPR assays. See text for more detail

Attempts to increase assay sensitivity of surface antibody-captured cells have included a classical sandwich-type approach. Once cells are captured a secondary cell-specific antibody is injected to increase the binding signal (*10;25*). This technique have however been found to require high antibody concentrations to achieve significant enhancement and as specific immunological reagents can be expensive and limited, this method is

not ideal. Another drawback of the sandwich-type technique is the requirement of two injections (cell-containing sample and secondary antibody), which increases analysis time.

The classical CM5 Biacore® chip has been used for most direct cell binding experiments and it has been suggested that the negatively charged dextran hydrogel would contribute to a repulsion of negatively charged cell walls (*36*). However, investigations using the alternative surface C1 sensor chip (a planar carboxymethylated surface with no dextran hydrogel), showed a decreased immobilisation of capture antibody, leading to decreased asssay sensitivity (*36*).

During immobilisation antibodies are attached to the surface in a random manner, which can decrease the surface cell-binding activity. To optimise cell-surface binding, researchers have attempted to control the orientation of immobilised antibody using capture on protein A/G surface. However, it has been found that a covalently immobilised antibody surface had a larger cell-binding capacity than when the same antibody was immobilised using a protein A-capture strategy (*25;53*). Furthermore, by using the protein A/G approach for orienting antibody, the sensor surface has to be replenished with cell-specific antibody after each measurement and regeneration round, which is time consuming.

Alternative subtractive inhibition assays for cell detection have been pursued. These techniques have been developed for analysis of viscous samples that cannot be introduced into the microfluidics of biosensors (*30;55*). The outline of a subtractive inhibition assay is seen in figure 1.10, exemplified by *Phytophthora infestans* detection. The original method was developed by Haines et al. (*30*) and involved the incubation of *Salmonella*-containing sample with pathogen specific pAb. After incubation the free pAb was separated from cell-bound pAb by the passage through a 0.22 µm filter. The remaining free pAb was subsequently quantified using a Biacore® instrument and a CM5-chip with anti-Fab as the ligand (*30*). Using this approach S*almonella* species could be detected at 10^4 cfu/ml. A further development of the method was

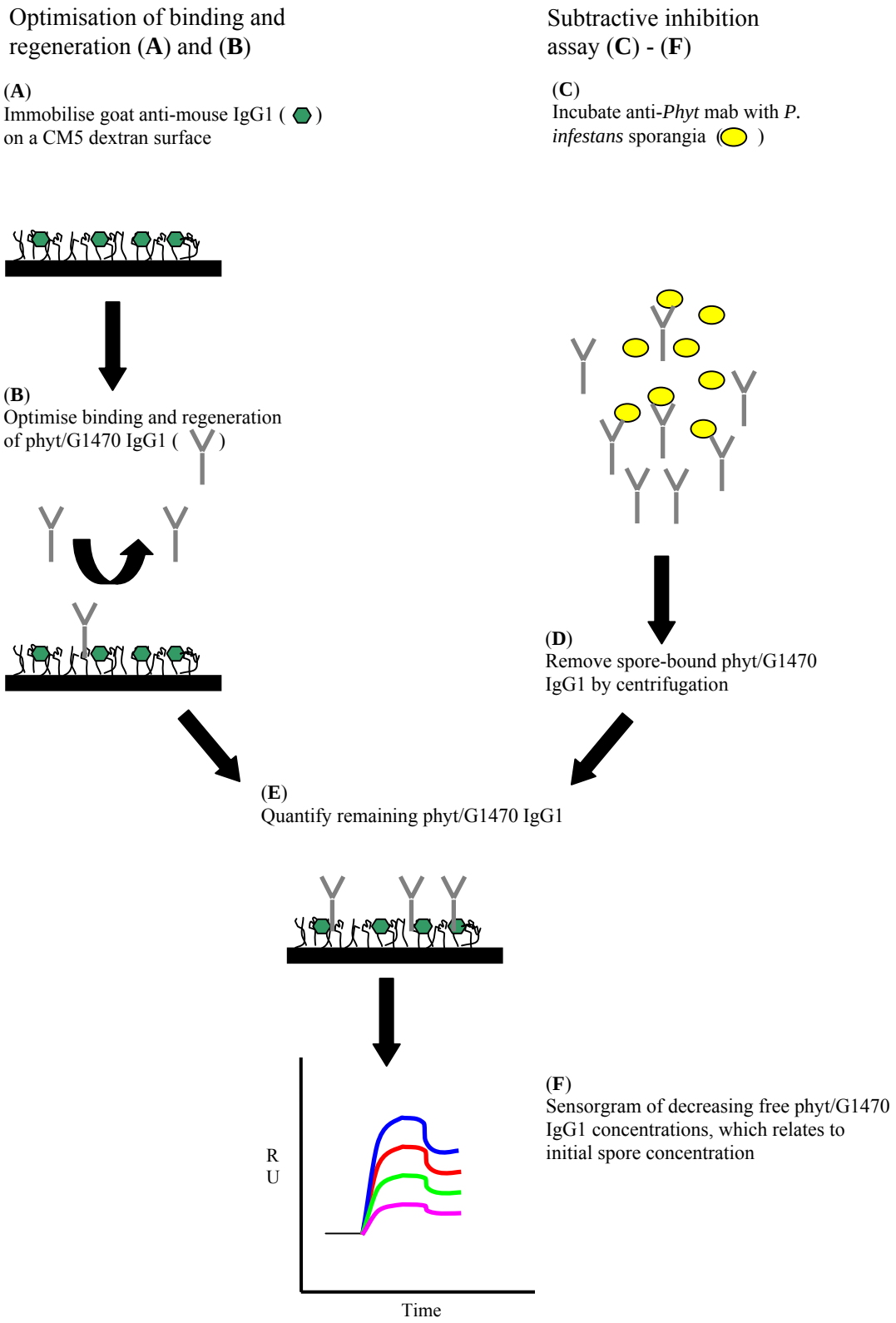

Figure 1.10: Subtractive inhibtion assay setup. **(A) - (B)** illustrates optimisation of regeneration. **(C)** – **(F)** shows the steps in a subtractive inhibition assay. See text for more detail

described by Leonard et al. (*55*), where *Listeria monocytogenes* were detected at 1 x 10^5 cfu/ml using a pAb raised against *L. monocytogenes*. In this approach the filtration step was substituted by a centrifugation step to fascilitate analysis of samples that are to viscous to be passed through filters (*55*). The method-improvement also meant that samples can be analysed in smaller volumes as the filter-step in the method from Haines et al. consumes sample. In unpublished work from van Knaben and co-workers S*almonella* species were detected at 10^6 cfu/ml (*6*). In this method an antibody targeting somatic antigens from *Salmonella* cell walls were incubated with *Salmonella* containing medium and as in the method of Leonard et al., unbound antibody was revovered by centrifugation. The antibody was quantified using sensor surface immobilised with *Salmonella* somatic antigens. All of these techniques have simplified *Salmonella* species and *L. monocytogenes* detection compared to direct cell capture assays. The major advantage of subtractive inhibition assays is that the pathogen is not introduced into the biosensor, which means that contamination problems are avoided. Furthermore, the assay is essentially a protein-protein interaction assay, meaning that the limitations for cell detection described above can be avoided.

1.5 *Puccinia striiformis* f.sp. *tritici*

1.5.1 Puccinia striiformis, a wheat pathogen

Wheat yellow rust (or stripe rust) caused by the biotrophic fungus *Puccinia striiformis* f.sp. *tritici*, is a common disease worldwide and have been reported in more than 60 countries on all continents except Antarctica (*15*). Yellow rust disease symptoms appear about 1 week after infection, and sporulation occur after 2 weeks, provided optimal temperature and moisture conditions are present. The symptom is seen as small, yellow- to orange-coloured rust pustules, which contain many thousand urediniospores. Urediniospores from *Puccinia* species are ovoid and large (18-45 μm in diameter) and many spores together appear as a yellow/orange-coloured powder (*56*). The disease is spread by the urediniospores and single spores can germinate and infect plants through the stomata and result in a lesion consisting of numerous pustules on the leaf surface. Moisture has been found to affect spore germination and infection. Therefore urediniospores require at least 3 h of continuous moisture (dew formation) on the surface of plants before germination occurs (*15*). During each generation (2-4 weeks), lesions may expand to some extent, but urediniospores are mostly spread by the wind to neighbouring leaves and plants, thereby establishing a focus in the field. Urediniospores can also spread over longer distances, whereby new foci are formed and after a suitable amount of time (a few generations) entire crop fields may be infected. New introductions of yellow rust disease by long-distance migration of spores have also been shown to take place (*40*). There is currently no evidence that *P. striiformis* completes a sexual reproductive cycle, as no alternate host has been identified, unlike many other cereal rusts (*15;40;48;81*).

1.5.2 Current methods for Puccinia striiformis detection

P. striiformis detection is currently performed by visual inspection of infected leaves on-site. This requires skilled personnel and is highly subjective. No immunological method exists for *P. striiformis* detection due to the lack of antibodies (however see paper 1 for antibody production). The only reported method for *P. striiformis* detection is a multiplex real-time PCR method (*24*). Using this method the simultaneous detection

of four different fungal species was possible in wheat, among them *P. striiformis*. The method was able to discriminate between the fungi by differences in their β-tubulin gene sequences.

1.6 *Phytophthora infestans*

1.6.1 Phytophthora infestans, an important pathogen worldwide

The genus *Phytophthora* represents a large group of plant pathogenic fungi responsible for crop losses in temperate and tropical climate (*45*). Most *Phytophthora* species damage roots, stem bases, bark and fruit. *P. infestans* is an exception in its ability to thrive in an aerial environment and attack foliage. Traditionally *Phytophthora* were grouped as a subdivision of the kingdom fungi, however currently the genera is grouped as a Oomycete and is more closely related to the brown and golden algae (*45*). *P. infestans* is the cause of the late blight diseases of potato and tomato. It is probably best known as the cause of the Irish potato famine of the 1840s resulting in up to one million dead people and a similar number of people emigrated to the rest of Europe and America (*46*).

P. infestans is dispersed by large wind-borne sporangia (diameter range of 12-23 μm), which are produced on branched hyphae (sporangiophores) that emerge from the stomata of infected leaves in humid conditions. Infections occur when sporangia land on plant tissue and release biflagellated zoospores, which encyst after a motile period and produce a germ tube which enters the plant through the stomata (*16*). Direct germination of sporangia is also possible. The pathogen penetrates the plant through the use of the tip of the germ tube. Intercellularly a biotrophic feeding relationships with plant cells is formed through haustoria. As the interaction proceeds new sporangia are formed on the plant surface. The aerial dispersal mechanisms and the ability to spread locally in drainage or irrigation water and persist as long-lived oospores in soil or planting material has resulted in a worldwide distribution of many *Phytophthora* species (*45;46*).

1.6.2 Current methods for Phytophthora infestans detection

There has been an increasing interest in *P. infestans* in recent years due to a dramatic increase in crop losses to late blight in many parts of the world. *P. infestans* are dependent on excess moisture for much of its life cycle and optimal temperature is also crucial for its growth. Detecting *P. infestans* at an early stage is important to minimise crop losses by crop spraying in a pre-emptive manner. Climate model decision support systems (DSS) are used to predict favourable conditions for pathogen reproduction and disease spread (*12*). However, differences in climate and local *P. infestans* populations illustrates that the models are not always useful in geographical regions other than the one in which they were developed (*18;37;50*).

Several laboratory-based *P. infestans* detection methods exist and they are all antibody- or nucleotide-based. A few nucleotide-based methods have been developed using different strategies for specific detection of *P. infestans*. These procedures involves PCR amplification of unique *P. infestans* 5.8S ribosomal DNA gene and/or internal transcribed spacers (ITS) (*47;71;83*). Furthermore, a Real-time quantitative PCR method has been developed targeting a GC-rich *P. infestans* species-specific DNA-region (*9*). Detection of *P. infestans* structures in infected plants can be achieved using available ELISA methods, however the detection is found to be specific to the *Phytophthora* genera only (*23;35;74*). In a recent study, Gough and co-workers developed phage-displayed scFv antibody fragment against surface exposed *P. infestans* antigens (*28*). These ScFv's could detect sporangia as well as zoospores but were also found to react with other *Phytophthora* species as well. Due to the lack of specific antibodies for *P. infestans* nucleotide-based PCR methods are currently most suitable for specific *P. infestans* detection. However, due to the longer analysis time of PCR-based methods (days, except the real-time PCR method which can be performed in hours, but requires specialised equipment) compared to ELISA methods (hours), a likely work-flow in analysis laboratories is to use time-saving ELISA tests for the pathogen genera identification followed by species identification by PCR if necessary.

1.7 Aims of the thesis

The aim of this thesis was to investigate the use of Surface Plasmon Resonance sensors for plant pathogen detection using monoclonal antibodies as the specific recognition element. Two plant pathogens were selected as model organisms in these studies, *P. striiformis* and *P. infestans*.

The specific aims were to:

- Produce and characterise mouse mAbs against urediniospores from *P. striiformis*
- Explore the diagnostic potential of the *P. striiformis* mAbs in a Surface Plasmon Resonance assay
- Use an existing mAb in the development of a Surface Plasmon Resonance assay for *P. infestans* sporangia detection

The following chapters (paper 1-3) will describe the results obtained in relation to the specific aims.

2. Paper 1

Monoclonal Antibodies for *Puccinia striiformis* Urediniospore Detection

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Summary

Two alternative monoclonal antibody-based assays for *Puccinia striiformis f.sp. tritici* (*Pst*) urediniospore detection are presented. The fungal pathogen *Pst* causes yellow rust disease in wheat plants leading to crop losses. The organism spreads by releasing wind dispersed urediniospores from infected plants. In this study a library of novel monoclonal antibodies (mAbs) was developed towards *Pst* urediniospores. Nine mAb-producing cell lines were fully cloned and their cross-reactivity characterised against a panel of airborne fungal spores representing genera commonly found in the same environment as *Pst*. Two specific mAbs were used to develop a competitive assay (*Pst* mAb4) and a subtractive inhibition assay (*Pst* mAb8). Standard curves for both assays had good intra- and interday reproducibility. The biosensor compatible subtractive inhibition assay had greater sensitivity with a detection limit of 1.5×10^5 spores/ml. Cross reactivity studies of *Pst* mAb8 in the subtractive inhibition assay, showed reaction with other *Puccinia* spores only, suggesting that common epitopes exist within this genus. The *Pst* mAb8 assay principle developed in this study have the potential to be implemented in future label-free in-the-field systems for *Pst* detection.

Introduction

Stripe or yellow rust of wheat is an important disease in wheat-growing regions of the world. The disease is caused by the biotrophic basidiomycete *Puccinia striiformis f.sp. tritici* (*Pst*). In years with severe infection, crop losses of up to 40 % have been reported for the most susceptible wheat varieties (Ullerup, 1991). *Pst* spreads by urediniospores, which are released from pustules on the leaf surface. A single infecting urediniospore may result in a lesion containing numerous pustules, which can be spread by wind dispersal to nearby plants resulting in so-called foci in the field. The urediniospores can also spread over longer distances, thereby generating new foci (Zadoks, 1961). It is extremely important to detect the disease at the early stages of the epidemic in order to optimise fungicide use. Specific and rapid molecular detection assays for *Pst* spores could be a useful supplement to the time consuming manual field monitoring and continuous inspection of infected leaves.

Nucleic acid-based techniques rely on detection of specific DNA sequences in the pathogen genome. The methods typically consist of sample extraction, a PCR amplification step and a product analysis step. The techniques are highly specific but generally time consuming and confined to a dedicated research laboratory environment (McCartney *et al.* 2003). Pathogen detection using specific antibodies offers the potential for more rapid analysis than nucleic acid-based techniques due to limited sample preparation (Ward *et al.* 2004; Werres & Steffens, 1994). This makes antibody based systems more attractive on-site, as rapid diagnosis of disease is essential to initiate correct control measures. Consequently, antibody-based dipstick methods and lateral flow devices are currently being used for fast on-site pathogen detection (Danks & Barker, 2000; Dewey *et al.* 1990; Ward *et al.* 2004). Detection of microorganisms using biosensors is a rapidly expanding area and has several advantages over existing dipstick and lateral flow devices, such as limited hands-on time, high-throughput screening and label-free detection. Immunobiosensors have been developed for the detection of several food and waterborne pathogens, such as *Salmonella* sp., *Escherichia coli* and *Listeria monocytogenes* (Bokken *et al.* 2003; Fratamico *et al.* 1998; Koubová *et al.* 2001; Leonard *et al.* 2004) and several biowarfare pathogens (Iqbal *et al.* 2000). Biosensors hold great potential in agricultural settings and

a continuous monitoring of *Pst* using an immunobiosensor would supply the farm manager with a rational decision making tool to optimise the use of agrochemicals.

Widely used biosensors, such as surface plasmon resonance (SPR) sensors have an efficient detection range only within 300 nm from the sensor surface. As the diameter of most bacteria and fungal pathogens exceed this range, assays using direct capture of cells on antibody surfaces have limited sensitivity (Leonard *et al.* 2004). To circumvent this limitation, indirect assays such as subtraction inhibition assays have been developed to increase biosensor sensitivity (Haines & Patel, 1995; Leonard *et al.* 2004). Plate-trapped antigen (PTA) and double antibody sandwich (DAS) assays are widely used for pathogen detection, but these are not compatible with biosensors.

The aim of this study was to develop *Pst* immunoassays for future implementation in label-free biosensors. No immunological method has been reported for *Pst* detection due to the lack of antibodies and in this study the production and application of novel mouse monoclonal antibodies (mAbs) specific to urediniospores from the *Puccinia* genus are described in the context of their diagnostic potential.

Materials and methods

Spore isolates

A 1:1 mixture of *Pst* urediniospores from isolates 16/02 and 66/02 (both of Danish origin) was used for immunisation and all other applications in this study. The following *Pst* isolates were used for specificity studies; 15/98, 17/97, 05/97 (Denmark), 92/4 (UK), k17a, k16b (Kazakstan), 87/11 (unknown origin), 98/603 (Italy), pak 19/04, pak 14/04 (Pakistan), E10a/03, E11a/03 (Eritrea). The following species were used for cross-reactivity studies; *Septoria nodorum, Botrytis cinerea, Penicillum roqueforti, Aspergillus versicolor, Fusarium culmorum, Epicoccum sp*., *Cladosporium cladosporioides*, *Cochliobolus sativus, Fusarium graminearum, Alternaria sp., Blumeria graminis, Tilletia tritici, Melampsora euphorbia, Miyagia pseudosphaeria, Cronartium ribicola, Puccinia recondita* and *Puccinia hordei*.

Production of monoclonal antibodies

Adult female BALB/c mice were initially injected intraperitoneally with 200 µl intact *Pst* urediniospores; 5 x 10^5 spores/ml in 50 % (v/v) Freunds Complete Adjuvant (FCA, DIFCO laboratories, Detroit, USA). Three weeks later the mice received another 200 µl injection of 5 x 10^5 spores/ml in 50 % (v/v) Freunds Incomplete Adjuvant (FIA, DIFCO laboratories, USA). Blood taken from the mice one week after each injection was analysed for immunoreactivity towards *Pst* urediniospores using PTA-ELISA (method described later) and subsequent immunisations were administered in 50 % FIA (v/v). After sufficient immunisations, mice received a booster injection (200 µl 5 x 10^5 spores/ml in 50 % FIA) and three days later they were sacrificed and the spleens were removed aseptically. The splenocytes were recovered and fused with X63Ag8.653 myeloma cells (Kearney *et al.* 1979) according to (Kohler & Milstein, 1975) modified as described in (Fonnesbech *et al.* 1993) and cultured as described previously (Lefkovits & Waldeman, 1979). Two mice were used in this study. Mouse 1 received 5 injections prior to fusion of the splenocytes. From the mouse 1 fusion one fully cloned and stable hybridoma was recovered. Mouse 2 received 9 injections (with approximately one month between the last four injections). From this fusion, eight fully cloned and stable hybridoma cell lines were obtained. Isotyping of the monoclonal antibodies (mAbs) was performed using rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM antibodies (DAKO-cytomation, Copenhagen,

Denmark). The murine IgM mAbs were purified from cell culture media using 1 ml HiTrap™ IgM Purification HP columns according to the Amersham Bioscience protocol (Hillerod, Denmark). Eluted fractions were analysed by SDS-PAGE for purity and by PTA-ELISA for antibody activity. Purified mAbs were buffer-exchanged into 50 mM Tris-HCl buffer pH 7.4 containing 200 mM NaCl, 10 mM glycine and 0.05 NaN_3 for storage at 4 °C.

Plate-trapped antigen ELISA (PTA-ELISA)

100 µl *Pst* spore suspension (5 x 10^5 spores/ml) in phosphate buffered saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) was added to each well in MaxiSorp™ plates (Nunc Glostrup, Denmark) and incubated for 16 hours at 37 °C. The wells were washed with PBS and blocked with 200 µl 1 % (w/v) BSA in PBS. Wells were incubated first with 100 µl hybridoma supernatant and then with 100 µl of Horse Radish Peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin (DAKO-cytomation) diluted 1/1000 in PBS containing 0.1 % (v/v) Tween20 (PBS-T). Both steps were performed for one hour at room temperature. Following each step, the plates were washed with PBS-T. Peroxidase-substrate (100 µl of [40 mM sodium acetate (pH 3.3), 3.2 mM $NaBO_2H_2O_23\ H_2O$, 2.8 % (w/v) 3,3´,5,5´-Tetramethylbenzidine (TMB)]) was added to each well and the reaction was stopped after 10 minutes by adding 100 µl 2 molar H_3PO_4. The absorbance was measured at 450 nm.

Extraction of soluble Pst spore components

A 2 x 10^8 *Pst* spores/ml suspension was washed for 2 hours in PBS on a vertical shaker to extract soluble components followed by sample centrifugation at 10,000 x g for 10 minutes. The liquid representing soluble spore components was removed and stored at -20 °C until further use and the washed spores were resuspended in the same volume of PBS and stored at -20 °C. Washed spores and soluble components were used to coat ELISA plates as described for PTA-ELISA. Hybridoma supernatants were added to wells at appropriate dilutions and incubated for 2 hours. The assay was developed as described for PTA-ELISA.

SDS-PAGE and Western blotting

20 µg dry spore sample was mixed with an equal amount of acid-washed sand and two steel grinding balls. *Pst* proteins were extracted using a Geno/Grinder at 1500 strokes/min for 30 seconds, a total of four times, followed by addition of 500 µl NuPAGE® 1 x LDS sample buffer (Invitrogen, Taastrup, Denmark), containing 20 mM DTT. The mixture was boiled for 20 min and centrifuged at 16,100 x g. The supernatant was isolated and extracted proteins were separated by SDS-PAGE (10 µl/well) using NuPAGE® 4-12% gradient gels (Invitrogen) and visualised by silverstaining. For Western blotting, proteins were blotted onto nitrocellulose membrane (0.45 µm, Sigma-Aldrich) with a semi-dry blot apparatus as described by the manufacturer (Hoefer Inc, San Francisco, USA). The blot was blocked with 1% dry milk (w/v) in TBS (20 mM Tris-HCl, 0.15 M NaCl, pH 7.4) and *Pst* mAb8 (0.5 µg/ml) diluted in TBS-T (TBS containing 0.05 % tween20 (v/v)) was added and incubated for one hour. Following incubation with AP-conjugated goat anti-mouse IgG + IgM diluted to 1/5000 in TBS-T for one hour, the membrane was washed and the bands visualised by adding AP-substrate (Sigma-FAST™, BCIP/NBT). After each step the blot was washed with TBS-T three times. As a control of specific binding, *Pst* mAb8 was substituted with a mouse mAb against human Chemotactic Protein-1 (Sigma, M-2420) at the same concentration.

Subtractive inhibition assay

Microtitre plates were coated with 0.1 µg rabbit anti-mouse IgM (Zymed Laboratories Inc., San Francisco, USA) in 100 µl PBS for 1 hour at 37 °C. The coated wells were washed with PBS and blocked with 200 µl 1 % (w/v) BSA in PBS. *Pst* spore standards were prepared by a three-fold dilution series (200 µl of each concentration) and *Pst* mAb8 (200 µl of 0.42 µg/ml) was added yielding a 0.21 µg/ml *Pst* mAb8 concentration. The mixture was incubated for one hour at 37 °C and inverted frequently to allow good mixing of spores and *Pst* mAb8. The spores were removed from the remaining free *Pst* mAb8 by sequential centrifugation as described previously (Leonard *et al.* 2004). The supernatants were carefully removed and added to ELISA plates in triplicate wells (100 µl/well) for each spore concentration. AP-conjugated goat anti-mouse IgG+IgM diluted 1/2500 in PBS-T was then added to the wells. Plates were washed with PBS-T and 100 µl of pNPP AP-substrate (Sigma-Aldrich) was added before incubation for 30 minutes in the dark at

37 °C. The plates were read at 405 nm. Unless otherwise stated all incubation steps were performed for one hour at 37 °C. The average response from the triplicate measurements (A) was divided by the average maximum response (Ao; antibody in PBS) to give the relation between free antibody and spore concentration (normalised values). Intraday (three assays in the same day) and interday (three assays on different days) validations were carried out to evaluate experimental variation. The detection limit was calculated as the lowest spore concentration resulting in >10% inhibition. For cross reactivity studies the assay was performed with serial dilutions of inhibiting antigen (different spore species) alongside the same dilutions with *Pst*. The percentage cross reactivity values were obtained with *Pst* mAb8 against the various spore species using the *Pst* concentration value at 10 % inhibition (IC_{10}) as a percentage of the cross reactant concentration giving the same decrease in signal.

Competition assay

Soluble antigen extracted from 5 x 10^6 spores/ml in PBS was prepared as above and used to coat microtiter plates (100 µl/well for 16 hours at 37 °C). Wells were blocked with 200 µl 1 % BSA (w/v) in PBS. *Pst* mAb4 (200 µl of 0.59 µg/ml) was incubated with 200 µl spore wash from different *Pst* concentrations (made by a three-fold dilution series of *Pst* spores) giving a final 0.295 µg/ml *Pst* mAb4 concentration. Each mixture was analysed in triplicate in spore wash coated microtitre wells (100 µl/well), where free and plate trapped antigen competed for *Pst* mAb4 binding for one hour at 37 °C, followed by a PBS-T wash. AP-conjugated goat anti-mouse IgG+IgM was added and the assay developed using pNPP substrate. All steps were performed for one hour unless otherwise stated. The average response from the triplicate measurements (A) was divided by the average maximum response (Ao; antibody in PBS) to establish the degree of competition (normalised values). Intraday and interday analysis were carried out to evaluate experimental variation. The detection limit was calculated as soluble antigen from the lowest spore concentration exhibiting >10% competition.

Results

Production and characterisation of antibody producing hybridomas

In this study we used intact *Pst* urediniospores as the immunogen for the production of specific mouse mAbs. The intact spores contained antigenic determinants, as blood collected from *Pst* immunised mice could be used for *Pst* detection in PTA-ELISA (data not shown). Two mice with the highest blood antibody titre were sacrificed and from the splenocyte fusions nine fully cloned antibody-producing cell lines, designated *Pst* mAb1-9, were obtained. The hybridomas were selected on the basis of a positive reaction with *Pst* urediniospore-coated microtitre wells. All cell lines were found to produce IgM-isotype antibodies. Antibody-containing culture supernatants from the mAb producing clones were tested by PTA-ELISA for cross-reactivity against spores from fungi representing genera commonly found in the same environment as *Pst*. These were *S. nodorum, B. cinerea, P. roqueforti, A. versicolor* and *F. culmorum* (table 2.1).

Antibody	*P. striiformis*	*S. nodorum*	*B. cinerea*	*P. roqueforti*	*A. versicolor*	*F. culmorum*	Control
Pst mAb1	1.11 ± 0.02	0.19 ± 0.02	0.37 ± 0.02	0.28 ± 0.01	0.42 ± 0.02	0.37 ± 0.02	0.24 ± 0.02
Pst mAb2	2.20 ± 0.05	0.19 ± 0.14	0.22 ± 0.03	0.20 ± 0.07	1.81 ± 0.08	1.04 ± 0.05	0.23 ± 0.43
Pst mAb3	1.35 ± 0.04	0.82 ± 0.05	1.54 ± 0.04	1.28 ± 0.06	1.73 ± 0.01	1.46 ± 0.04	0.23 ± 0.05
Pst mAb4	2.66 ± 0.23	0.01 ± 0.00	0.03 ± 0.02	0.02 ± 0.00	0.15 ± 0.07	0.05 ± 0.01	0.00 ± 0.00
Pst mAb5	1.67 ± 0.01	0.04 ± 0.02	0.17 ± 0.01	0.08 ± 0.01	1.12 ± 0.01	0.78 ± 0.08	0.02 ± 0.01
Pst mAb6	0.84 ± 0.03	0.02 ± 0.00	0.59 ± 0.02	0.03 ± 0.00	0.69 ± 0.03	0.16 ± 0.01	0.00 ± 0.00
Pst mAb7	0.76 ± 0.02	0.12 ± 0.00	0.57 ± 0.03	0.06 ± 0.01	0.36 ± 0.03	0.11 ± 0.01	0.01 ± 0.00
Pst mAb8	1.29 ± 0.05	0.03 ± 0.01	0.05 ± 0.01	0.02 ± 0.05	0.33 ± 0.02	0.02 ± 0.00	0.01 ± 0.00
Pst mAb9	1.07 ± 0.05	0.05 ± 0.06	0.03 ± 0.00	0.01 ± 0.00	0.27 ± 0.01	0.02 ± 0.00	0.01 ± 0.00

Table 2.1: Cross-reactivity of mAbs determined by PTA-ELISA. Undiluted hybridoma supernatant from individual clones were tested for mAb-binding to approximately same amounts of plate-trapped spores from *Pst* and fungi representing genera commonly found in the same environment as *Pst*. For visualisation a peroxidase-conjugated rabbit-anti-mouse immunoglobulin was added and the assay developed. Absorbance values were measured at 450 nm. The results shown are means and standard deviations in quadruple measurements. The control represents an overnight coat with PBS only.

Pst mAb1, 2, 3, 5, 6 and 7 reacted with some or all of the non-target fungal spores. *Pst* mAb4, mAb8 and mAb9 however, showed positive reactions towards *Pst* and a response at least three times higher than against the non-target spores. *Pst* mAb4 and *Pst* mAb8 were selected for further analysis. The *Pst* mAb4 and *Pst* mAb8 were not isolate specific as they both reacted with 12 different isolates of *Pst* (15/98, 17/97, 05/97, 92/4, k17a, k16b, 87/11, 98/603, pak 19/04, pak 14/04, E10a/03 and E11a/03) collected in various countries (data not shown). This indicates that *Pst* mAb4 and *Pst* mAb8 has the potential to detect any *Pst* isolate, regardless of continental origin, but further investigations are needed before this conclusion can be made.

Epitope characterisation

Periodate oxidation of intact *Pst* spores did not have any effect on *Pst* mAb4 and *Pst* mAb8 binding to the spore antigens, which suggest that glycans are not part of the epitopes for these two mAbs (data not shown). Soluble components were extracted from urediniospore surfaces by a PBS buffer wash and the binding of *Pst* mAb4 and *Pst* mAb8 to soluble (*Pst* surface wash) and insoluble (washed *Pst* spores) material was tested. *Pst* mAb4 did not exhibit any reaction with washed spores but a high reactivity was observed with *Pst* surface wash, thereby illustrating that the *Pst* mAb4 protein antigen is soluble (figure 2.1A). In contrast, *Pst* mAb8 reacted more strongly with washed *Pst* spores, revealing that the *Pst* mAb8 antigen is less soluble and probably an integral part of the spore surface (figure 2.1B). *Pst* proteins were extracted and separated by SDS-PAGE and figure 2.2A shows the protein profile of the extract. Multiple protein bands are observed mostly in the range of 19-191 kDa. By Western blotting, using *Pst* mAb8 as detection antibody, two bands were observed, an intense band at ~39 kDa and a weaker band at ~20 kDa (figure 2.2B, lane 3). By comparison to the control experiment, the band at ~20 kDa could be attributed to non-specific binding caused by the AP-labeled conjugate, (figure 2.2B lane 2). The protein band at ~39 kDa, however, was not visible in the control experiment, therefore representing a specific target for *Pst* mAb8. Similar Western blotting experiments with *Pst* mAb4 were not successful. It is concluded that the antigen targeted by *Pst* mAb4 is easily solubilised and that *Pst* mAb8 interacts with a less soluble ~39 kDa protein part of the spore surface.

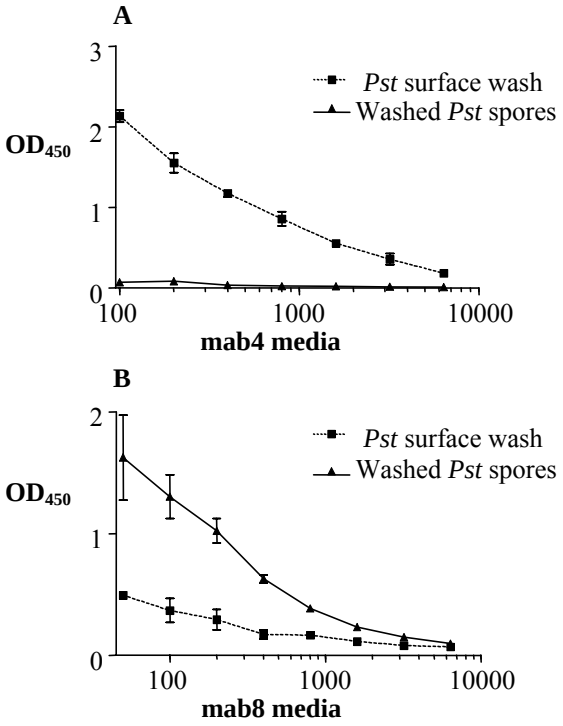

Figure 2.1: The binding of *Pst* mAb's in diluted hybridoma media to soluble components and washed *Pst* spores. *Pst* mAb4 reactivity with *Pst* spores was absent following a PBS wash of the spores, suggesting that the antigen is soluble (panel A). *Pst* mAb8 reacted better with washed spores, indicating that the antigen is less suluble and associated with the spore surface (panel B). All results represent mean and standard deviations from triplicate measurements.

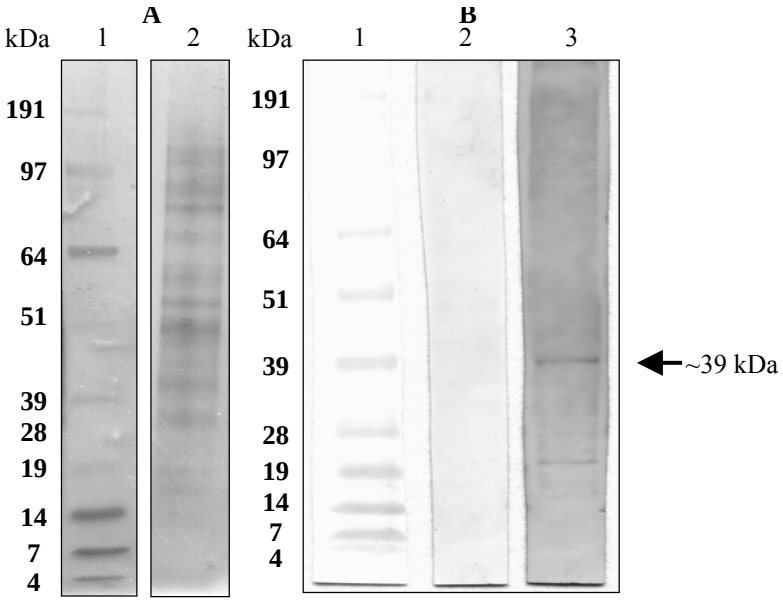

Figure 2.2: SDS-PAGE analysis and Western blotting. Panel A: (1) Seeblue Plus2® pre-stained molecular weight standard (Invitrogen), (2) silverstaining of a full *Pst* protein extract showing multiple protein bands of various molecular weights. Panel B: (1) Seeblue Plus2® pre-stained molecular weight standard, (2) Western blot developed using control mAb, (3) Western blot developed using *Pst* mAb8. *Pst* mAb8 binds two protein bands at ~20 kDa and ~39 kDa. The staining of the ~20 kDa band could be attributed to non-specific binding of the AP-conjugate as the same band was seen in control experiments. The staining of the ~39 kDa protein (arrow) represents a specific interaction with *Pst* mAb8.

Assay development

Two different assays for *Pst* detection were developed on the basis of the different types of antigen recognised by *Pst* mAb4 and *Pst* mAb8; a competitive assay for detection of the soluble antigen using *Pst* mAb4 and a subtractive inhibition assay for detection of intact spores with *Pst* mAb8. Normalised values (A/Ao) for each spore standard concentration were plotted against the spore concentration to obtain calibration curves for each assay in the range of 1.6 x 10^4 to 1.1 x 10^8 spores/ml, as depicted in figure 2.3. The stability of both assays were analysed by interday and intraday analysis and good coefficients of variation (CV's) were obtained for all spore concentration standards (table 2.2). From the calibration curves it was concluded that the subtractive inhibition assay had a broader dynamic range for *Pst* detection than the competitive assay. Furthermore, the detection limit for the subtractive inhibition assay was 1.5 x 10^5 spores/ml, whereas the detection limit for the competitive assay was much higher (1.2 x 10^7 spores/ml).

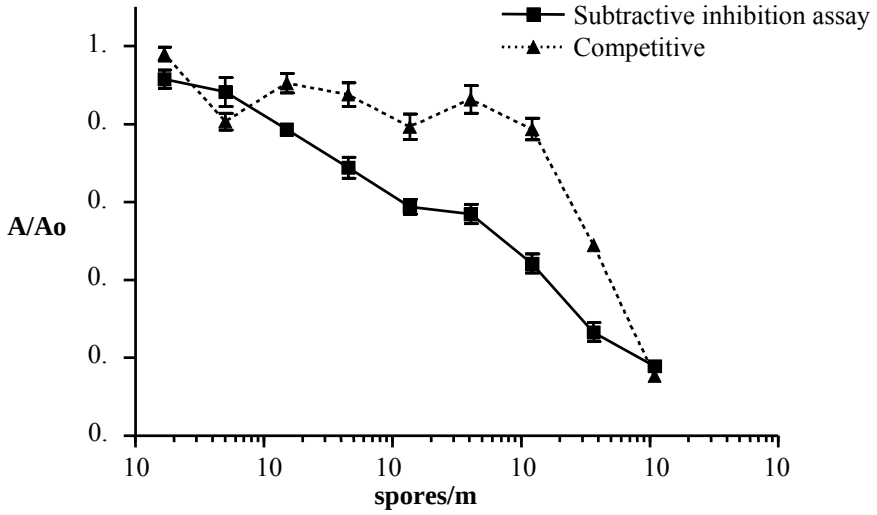

Figure 2.3: Interday calibration curves from the competitive and inhibition assays. The data presented are mean values and standard deviations from 3 independent assays with triplicate values. The subtractive inhibition assay had a LOD of 1.51 x 10^5 spores/ml, whereas the competitive assay had a LOD of 1.36 x 10^6 spores/ml.

	Inhibition assay		Competitive assay	
Spore concentration (spores/ml)	Intraday CV's (%)	Interday CV's (%)	Intraday CV's (%)	Interday CV's (%)
1.10×10^8	6.85	11.54	3.34	10.47
3.67×10^7	6.73	15.83	10.59	15.65
1.22×10^7	6.74	9.59	6.15	11.89
4.07×10^6	6.22	7.52	7.21	15.84
1.36×10^6	8.21	5.66	7.14	17.32
4.53×10^5	9.71	6.77	5.98	13.67
1.51×10^5	3.57	0.55	14.68	13.01
5.03×10^4	9.35	7.35	4.57	11.64
1.68×10^4	2.29	4.46	3.09	15.92

Table 2.2: Validation data from the inhibition assay and the competitive assay. Data represents three assays performed on the same day (intraday) and on three different days (interday). Coefficients of variation (CV's) are defined as standard deviations divided by the mean from each assay (n=3) x 100 %

Cross-reactivity evaluation of the subtractive inhibition assay

The subtractive inhibition assay (incorporating *Pst* mAb8) was challenged with a panel of spores derived from seven common fungal species and five rust species and the percent cross-reactivities calculated from the inhibition profiles obtained (table 2.3). In general, limited cross-reactivity was found towards the non-rust species; *Epicoccum sp*., *C. cladosporioides*, *C. sativus, F. graminearum, Alternaria* sp.*, B. graminis* and *T. tritici*. Analysis of the inhibition profiles for the rust species revealed low cross-reactivity towards *M. euphorbia* and only partial cross-reactivity was observed towards *M. pseudosphaeria* and *C. ribicola*. However, *Pst* mAb8 exhibited a high degree of cross-reactivity towards both *P. recondita* and *P. hordei*, suggesting that *Pst* mAb8 targets epitopes, that are present in other *Puccinia* species also.

Spore species	%-cross reactivity
Epicoccum sp.	1.3
Cladosporium cladosporioides	1
Alternaria sp.	2
Cochliobolus sativus	0.8
Fusarium graminearum	0.8
Blumeria graminis	0.7
Miyagia pseudosphaeria	8.8
Cronartium ribicola	10.5
Tilletia tritici	2.7
Melampsora euphorbia	1.1
Puccinia recondita	81.5
Puccinia hordei	72

Table 2.3: Cross-reactivity of *Pst* mab8 with non-target spores. Inhibition curves were generated for each spore species by assaying 3-fold dilution series in triplicate. The concentration where 10 % inhibition (IC10) was observed was used to calculate % cross-reactivity (CR) using the expression; CR = (C/C*) x 100 %, where C and C* are the IC10-values for *Pst* and the reference spore, respectively.

Discussion

In this study unique mAbs using intact *Pst* spores as the immunogen were developed. The mAbs were further applied in novel antibody based assays for *Pst* spore detection. Different strategies are used in the production of antibodies against fungal pathogens. Antigens have included soluble extracellular glycans/glycoproteins, fragments from spore cell walls and intact spores or mycelia (Werres & Steffens, 1994). The type of antigen used for antibody development may be very application dependent. As our long term aim is to detect intact spores in air samples, these were used as immunogen and our splenocyte fusions resulted in nine IgM-producing hybridoma cell lines. Immunisation schemes using fungal antigens often result in IgM-producing hybridomas: 4 IgM's out of 4 mAbs (Bermingham et al., 1995), 1/1 (Fuhrmann *et al.* 1992), 26/35 (Koistinen *et al.* 2000), 7/9 (Salinas & Schots, 1994), 7/12 (Schmechel et al. 1997), 5/5 (Schmechel *et al.* 2003), 35/46 (Schmechel et al., 2005), 5/6 (Schmechel *et al.* 2006) and 1/1 (Thornton *et al.* 2002). Results from an earlier study indicate that the type of antigen used for immunisation can be important for the antibody isotype produced, as spore fragments gave a higher number of IgG producing hybridomas compared to intact spores (Xia *et al.* 1992). It is generally accepted that IgM antibodies frequently recognise repetitive epitopes such as those expressed by bacterial cell-wall polysaccharides and it is likely that immunodominant repetitive epitopes are present on *Pst* spores. IgM antibodies are often of low affinity (Kd in the μM range) and therefore usually neglected as tools in immunoassays if IgG subtype mAbs are available. We speculate that the avidity of the pentameric IgM binding to *Pst* repetitive epitopes may increase the total binding strength compared to IgG's. This could mean that IgM-producing hybridomas were preferentially enriched and selected during the mAb screening using plate-trapped intact spores. The IgM's produced in the present study were fully functional in the formats used, but it is likely that a revision of the immunisation and screening procedure, e.g. using urediniospore fragments, could lead to generation of IgG-mAb-producing clones.

The components of the spore surface of *Pst* are largely unknown and knowledge of the nature of the antigenic determinants is limited. We investigated the effect of periodate oxidation on the epitopes and found that this did not affect *Pst* mAb4 and *Pst* mAb8 antigen binding. This indicated that glycans do not contribute to the *Pst* mAb4 and *Pst* mAb8 epitopes. By Western blotting a ~39 kDa protein was identified as

the target for *Pst* mAb8. Similar Western blotting analyses using *Pst* mAb4 were not successful suggesting that either the antigen was not extracted in sufficient amounts or that the *Pst* mAb4 recognises a conformational epitope that is irreversibly destroyed during SDS-PAGE.

Both *Pst* mAb4 and *Pst* mAb8 detected *Pst* isolates from a wide geographic origin. This suggest that the antigens targeted by *Pst* mAb4 and *Pst* mAb8 are common components of *Pst* isolates worldwide. A mixture of two genetically different (Mogens S. Hovmøller, personal communication) Danish *Pst* isolates (*Pst* 16/02 and *Pst* 66/02) were used for the immunisation and screening. The mAbs have therefore targeted epitopes that are common to both *Pst* 16/02 and *Pst* 66/02, which are also present in all isolates tested. Furthermore, *Pst* mAb8 successfully recognised *P. recondita* and *P. hordei*, which is in agreement with previous findings, that mAbs are often specific to the genus level only (Bossi & Dewey, 1992; Fuhrmann *et al.* 1992; Schmechel *et al.* 2003; Thornton *et al.* 2002).

The two immunoassays developed in this study can be used for quantification of *Pst*. The assays had similar total analysis time and can be performed within a normal working day. Both assay calibration curves had good CV's, ranging from 0.55-15.83 % (subtractive inhibition assay) and 3.09-17.32 % (competitive assay). The subtractive inhibition assay could be used for *Pst* detection in the range 1.6×10^4 to 1×10^8 spores/ml and the detection limit was 1.5×10^5 spores/ml. A similar subtractive inhibition ELISA have been reported for detection of the bacteria *Listeria monocytogenes* with a detection limit of 1×10^5 cells/ml (Leonard *et al.* 2004). No reference subtractive inhibition assays exist concerning fungal detection, but the detection limit for the *Pst* subtractive inhibition assay is similar to that of 1×10^5 spores/ml, which have been reported for a *Trichoderma harzianum* spore PTA-ELISA using a mouse IgM mAb (Thornton & Dewey, 1996). This supports the merits of the *Pst* subtractive inhibition assay in terms of sensitivity.

As yellow rust disease is distributed world wide, the subtractive inhibition assay can be used in laboratories where quantification of urediniospores is needed. Similar subtractive inhibition biosensors have been successful in detection of food-borne bacteria (Haines & Patel, 1995; Leonard *et al.* 2004) and our future work includes the implementation of the assay in label-free biosensors.

Acknowledgements

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3. PAPER 2

Detection of Fungal Spores Using a Generic Surface Plasmon Resonance Immunoassay

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Abstract

This paper describes a biosensor-based method for detection of fungal spores using Surface Plasmon Resonance (SPR). The approach involves the use of a mouse monoclonal antibody (*Pst* mAb8) and a SPR sensor for label-free detection of urediniospores from the model organism *Puccinia striiformis f.sp. tritici* (*Pst*). In the subtractive inhibition assay, urediniospores and *Pst* mAb8 were mixed, urediniospore-bound *Pst* mAb8 removed by centrifugation and the remaining *Pst* mAb8 quantified using the SPR sensor. Assay conditions were optimised and a detection limit of 3.1×10^5 urediniospores/ml was achieved. Spiked *Pst* samples were further examined in a background of a related spore and it was found that *Pst* quantification was possible in this mixture. This study represent the first use of SPR technology for fungal spore detection as well as the first report of a successful biosensor-based detection strategy for *Pst*.

Keywords

Surface Plasmon Resonance, immunosensor, fungal spore, *Puccinia striiformis*, monoclonal antibody

1. Introduction

Fungi are found in a wide range of environments and most species are spread by airborne spores. Fungi can cause allergic reactions and serious diseases in humans and animals (Horner et al., 1995). In agricultural plant production, fungal diseases can have a great impact on crop yield and quality (Yarden et al., 2003). Knowledge of the exact location and time of occurrence of diseases within the field may expedite the implementation of suitable control strategies for protection and thus reduce the use of agrochemicals. Currently, crop disease status has to be monitored by repeated visual screening for pests and pathogens, which is time-consuming, expensive and requires skilled personnel. The development of fast and specific screening methods is, therefore, a priority. Several molecular detection systems for fungal spore identification exist. These laboratory-based label-dependent systems are based primarily on specific nucleotide primers used for Polymerase Chain Reaction (PCR) amplification of DNA regions or on antibodies reacting specifically with the pathogens in question (Ward et al., 2004). Although nucleic acid-based detection systems are likely more specific for individual species, antibody-based methods are often conducive to rapid analysis, since limited sample extraction is needed. Antibody detection systems are therefore more suited for future implementation in label-free immunosensors that could be mounted on platforms for on-site continuous screening for various pathogens.

The surface plasmon resonance (SPR) detection principle is utilised in many optical biosensor systems for the study of 'real-time' molecular interactions. Several commercial SPR sensors are now available and have been used for detection of various analytes ranging from small molecules to intact cells; for reviews see (Baird and Myszka, 2001; Karlsson, 2004; Leonard et al., 2003). The Biacore® system uses SPR to detect the binding of analytes to immobilised ligands. The sensor system measures refractive index changes on or near the sensor surface, which is proportional to mass changes occurring upon analyte binding. Biacore® and other SPR immunosensors, have been used for the detection of bacterial cells by their capture onto immobilised antibodies on sensor chip surfaces (Fratamico et al., 1998; Bokken et al., 2003; Koubová et al., 2001). SPR assays based on the surface capture of intact cells have limited sensitivity for the following three main reasons: (i) Under total internal reflection, the penetration depth of the electromagnetic field wave (evanescent field) in the SPR system is approximately 300 nm and consequently only refractive index

changes occurring within this distance will be recorded by the SPR sensor. This decreases assay sensitivity as only a small part of a cell is within the 300 nm range. Attempts have been made to increase the penetration depth of the evanescent field by using special kinds of surface plasmons, in so-called long-range surface plasmon resonance (LRSPR) sensors (Matsubara et al., 1990), but until now no commercial instruments are available (Homola, 2003). (ii) Sensor chips consist of a dextran strand matrix attached covalently to the gold surface. Dextran strands are functionalised along their entire length during ligand immobilisation, however intact cells are not able to penetrate the matrix layer, leading to many unoccupied antigen binding sites. (iii) Direct cell binding requires that the cell-antibody binding avidity must be high to resist the hydrodynamic forces that act on the cells in the micro flow channels.

Several successful attempts have been made to improve the inherent low sensitivity of SPR cell detection. Increased sensitivity is often obtained by sandwich-type assays, where a second cell specific antibody is used to amplify the signal (Bokken et al., 2003; Fratamico et al., 1998). Indirect subtractive inhibition assays have also proved sensitive in cell detection (Haines and Patel, 1995; Leonard et al., 2004).

In the present study, urediniospores from *Puccinia striiformis f.sp. tritici* (*Pst)* were chosen as a model system for the development of a SPR fungal spore biosensor. The organism causes yellow rust disease, which is one of the most important diseases of wheat worldwide (Chen, 2005). The disease spreads by wind-dispersed urediniospores, leading to the development of new foci of infection in the field (Hovmoller et al., 2002). Urediniospores from *Puccinia* species are ovoid and large (18-45 μm in diameter) and their surface is covered with spines typically about 1 μm in length (Littlefield, 2000). Direct spore capture assays via immobilised antibodies would have limited sensitivity as the spore diameter far exceeds the 300 nm range. Furthermore, due to the large size of urediniospores, direct injection into Biacore® micro flow channels could block the microfluidic path and potentially damage the instrument hardware. At present, SPR detection based on direct fungal spore capture on antibody-immobilised surfaces has not been reported, and other strategies must be pursued. The subtractive inhibition approach simplifies detection of microorganisms as only a protein-protein interaction is measured and in general increased sensitivity has been achieved (Haines and Patel, 1995; Leonard et al., 2004).

In this study a subtractive inhibition assay for *Pst* urediniospore detection is evaluated. The method is based upon the specific interaction of a monoclonal antibody and urediniospores in solution. The following quantification of remaining free unbound antibody by SPR can thereby be related to spore concentration.

2. Materials and methods

2.1 Pst Spore production

A 1:1 mixture of urediniospores from *Pst* isolates, 16/02 and 66/02, were used in this study. The spores were cultivated and harvested on wheat plants and stored as previously described (Justesen et al., 2002).

2.2 Pst Antibody

A mouse monoclonal IgM antibody raised against intact *Pst* urediniospores, designated *Pst* mAb8 (Skottrup et al.) was used in this study. The antibody was purified from cell culture medium using HiTrap IgM Purification HP columns according to the Amersham Bioscience protocol (Uppsala, Sweden) and antibody purity and activity were verified by SDS-PAGE analysis and ELISA.

2.3 Antibody immobilisation

Analysis was carried out on a Biacore® 3000 sensor (Uppsala, Sweden) using a filtered and degassed HBS running buffer (10 mM HEPES, 150 mM NaCl, 3.8 mM EDTA, 0.05% (v/v) Tween). Sensor chip CM5 (Biacore®) dextran surfaces were used throughout the study. The optimal pH for immobilisation of commercial rabbit anti-mouse IgM polyclonal antibody (Zymed laboratories Inc., San Francisco, USA) was determined by diluting the antibody (10 µg/ml) in 10 mM sodium acetate buffers with varying pH (3.8-5.0). A 20 µl volume of the antibody preparation at each pH was injected (flowrate 5 µl/min) over a blank flow cell in a CM5 chip, and pH 4.5 was found to be optimal for immobilisation. The CM5 chip was first activated by mixing 50 µl of 100 mM N-hydroxysuccinimide (NHS) with 50 µl of 400 mM N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and injecting 70 µl at 10 µl/min over the dextran surface of a CM5 chip. Rabbit anti-mouse IgM was immobilised using a 100 µg/ml solution diluted in 10 mM sodium acetate buffer, pH 4.5, which was injected at a flowrate of 10 µl/min over the activated dextran surface for 12.5 minutes. Approximately 9000 response units (RU) of rabbit anti-mouse IgM was immobilised onto the activated dextran. Unreacted sites were subsequently deactivated by injecting 1 M ethanolamine, pH 8.5 (Biacore®), at 10 µl/min for 7 min.

2.4 Surface regeneration

The stability of the anti-mouse IgM surface was investigated by repeated cycles of *Pst* mAb8 injections and surface regenerations. 40 µl samples of 5 µg/ml *Pst* mAb8 in PBS (20 mM sodium phosphate, 150 mM

NaCl, pH 7.4) were injected at a flowrate of 5 µl/min. The surface was effectively regenerated using 50 µl of 20 mM HCl at a flowrate of 10 µl/min.

2.4 Assay design

Dilution series of *Pst* urediniospores in PBS was performed, thereby obtaining *Pst* urediniospore concentration standards in 300 µl PBS. 300 µl of purified *Pst* mAb8 (10 µg/ml dilution) in PBS was added to each urediniospore standard, yielding a *Pst* mAb8 final working concentration of 5 µg/ml. The mixtures were incubated at 37 °C for 30 min and inverted frequently to allow optimal urediniospore and antibody contact. The urediniospore-bound antibodies were separated from free antibody by sequential centrifugation as described previously (Leonard et al., 2004). The supernatants were carefully removed without touching the urediniospore pellet and 40 µl of each supernatant was assayed randomly in duplicate by automated analysis using the Biacore® 3000 instrument. The samples were injected over the CM5 chip surface at a flow rate of 5 µl/min and the resulting response unit change was measured at the end of each injection. Following each sample injection the surface was regenerated using 20 mM HCl as described. The assay was repeated on three separate occasions and the data were analysed by taking the mean value from duplicate measurements at each urediniospore concentration (R) and a mean blank sample response (Ro). The latter consisted of *Pst* mAb8 in PBS only. Normalised values were calculated by dividing mean values by the mean blank response (R/Ro), thereby obtaining the degree of inhibition for each urediniospore concentration. A calibration curve was constructed to attain the degree of inhibition as a function of the urediniospore concentration and the detection limit was calculated as the lowest point exhibiting >10% inhibition.

2.5 Cross reactivity studies

The non-rust fungus *Tilletia tritici* and three rust species, *Melampsora euphorbia, Miyagia pseudosphaeria* and *Cronartium ribicola* were tested in the subtractive inhibition assay. A concentration of 5 x 10^6 spores/ml were used for each species (300 µl) and 300 µl µl of purified *Pst* mAb8 (10 µg/ml dilution) in PBS was added. The samples were analysed alongside *Pst* and the assay was performed as described above. To study *Pst* detection in the presence of another spore species, a new CM5 chip was immobilised as described above. *Pst* was spiked into 5 x 10^6 *M. euphorbia* spores/ml at concentrations of 10^4, 10^5 and 10^6 urediniospores/ml, *Pst* mAb8 was added and the samples analysed as above in triplicate for each sample.

3. Results and discussion

A subtractive inhibition assay for urediniospore detection based on a *Puccinia spp.* monoclonal antibody (*Pst* mAb8) and a Biacore® 3000 instrument was developed.

Functionalisation of a sensor surface with appropiate capture ligand is a prerequisite for specific detection of biomolecules. *Pst* mAb8 used in this study was a mouse IgM and a CM5 chip was therefore immobilised with a polyclonal rabbit anti-mouse IgM immunoglobulin. The activity and specificity of the surface was verified by simultaneously injecting *Pst* mAb8 over a blank dextran surface and the functionalised surface. No significant binding was observed on the blank surface compared to the rabbit anti-mouse IgM surface (data not shown). Regeneration was optimised and a single injection of 50 µl 20 mM HCl was sufficient for surface regeneration (data not shown).

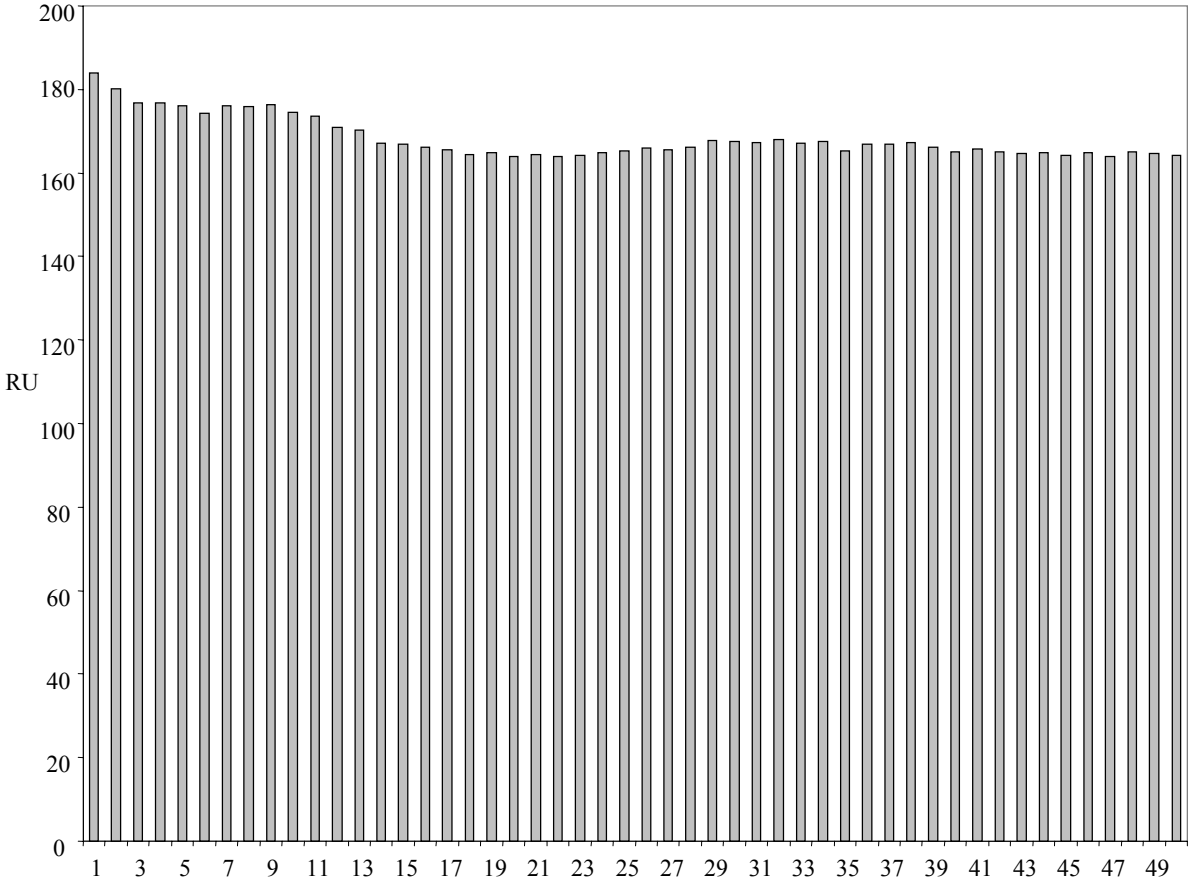

Figure 3.1: Surface stability test. Repeated *Pst* mAb8 injections (40 µl of 5 µg/ml in PBS) and surface regenerations using 50 ul of 20 mM HCl at a flowrate of 10 µl/min. The long term stability of the sensor surface was confirmed with only a 10.7 % decrease in activity from the first binding cycle (183.9 RU) to the last (164.2 RU). After approximately 15 regenerations the sensor surface activity stabilised whereafter it was used in the subtractive inhibition assay and cross-reactivity studies.

To investigate surface stability repeated *Pst* mAb8 injections and 20 mM HCl regeneration cycles were performed (figure 1). After approximately 15 regenerations the chip surface stabilised and following 50 injections, a decrease in surface activity of 10.7 % was observed.

The chip was used in the subtractive inhibition assay and for cross-reactivity studies. During these experiments the surface activity of the chip did not decrease more than 20 %, which is the recommended cut-off value that ensures validity in Biacore assays with repeated surface regenerations (Wong et al., 1997).

For *Pst* detection urediniospore standards were serially diluted in PBS and *Pst* mAb8 was added to each urediniospore concentration standard. After centrifugation, each urediniospore concentration standard was analysed in duplicate in Biacore 3000®. An overlay plot from typical experiments shows that responses are inversely proportional to the concentration of the inhibiting urediniospores, thereby verifying the biosensing principle (figure 2A). A calibration curve using normalised data (R/Ro) from three separate assays, plotted against the urediniospore concentration, was constructed (figure 2B). The coefficients of variation (CV) were calculated for the individual urediniospore concentration standards ranging between 1.74-6.92 % and from the data and the calibration curve, a detection limit of 3.1×10^5 urediniospores/ml was achieved.

The ability of *Pst* mAb8 to discriminate between *Pst* and related rust fungi was investigated. When solutions of several rust species (and one non-rust species, *T. tritici*) were tested in the *Pst* mAb8 assay (5×10^6 spores/ml) weak reactivity was found only with *C. ribicola* and *M. euphorbia* (figure 3A). The interaction of *Pst* mAb8 with these species is probably binding caused by the high spore concentration used. To further investigate *Pst* detection in the presence of the weak cross-reactant *M. euphorbia* at 5×10^6 spores/ml, samples were spiked with *Pst* in different concentrations (10^4, 10^5 and 10^6 urediniospores/ml). As seen from figure 3B, the R/Ro values decreased in response to increasing *Pst* urediniospore concentrations, thereby demonstrating that *Pst* mAb8 is capable of specifically detecting *Pst*.

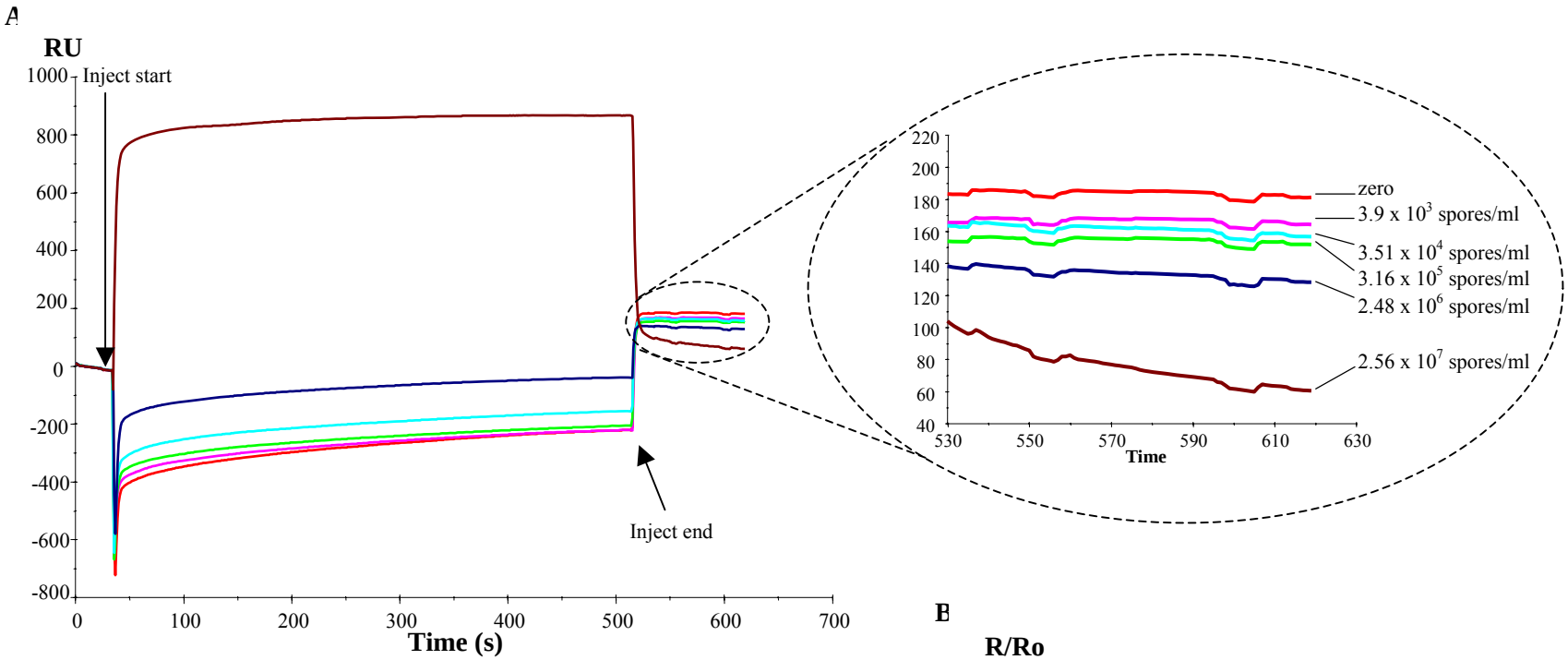

Figure 3.2: Overlay plot and calibration curve. Panel A: Overlay plot demonstrating the decrease in binding of *Pst* mAb8 to the anti-mouse IgM surface with increasing *Pst* urediniospore concentrations. Panel B: The mean binding response at each cell concentration (*R*) was divided by the mean antibody binding response determined in the absence *Pst* urediniospores (*Ro*) to give a normalised binding response (*R*/*Ro*). A calibration curve was constructed from the mean of R/Ro-values and corresponding standard deviations (SD) from three independent assays. A detection limit of 3.1 x 10^5 urediniospores/ml was achieved. Coefficients of variation (CV = SD/(R/Ro mean value)) for the individual concentration standards ranged between 1.74 % and 6.92 %.

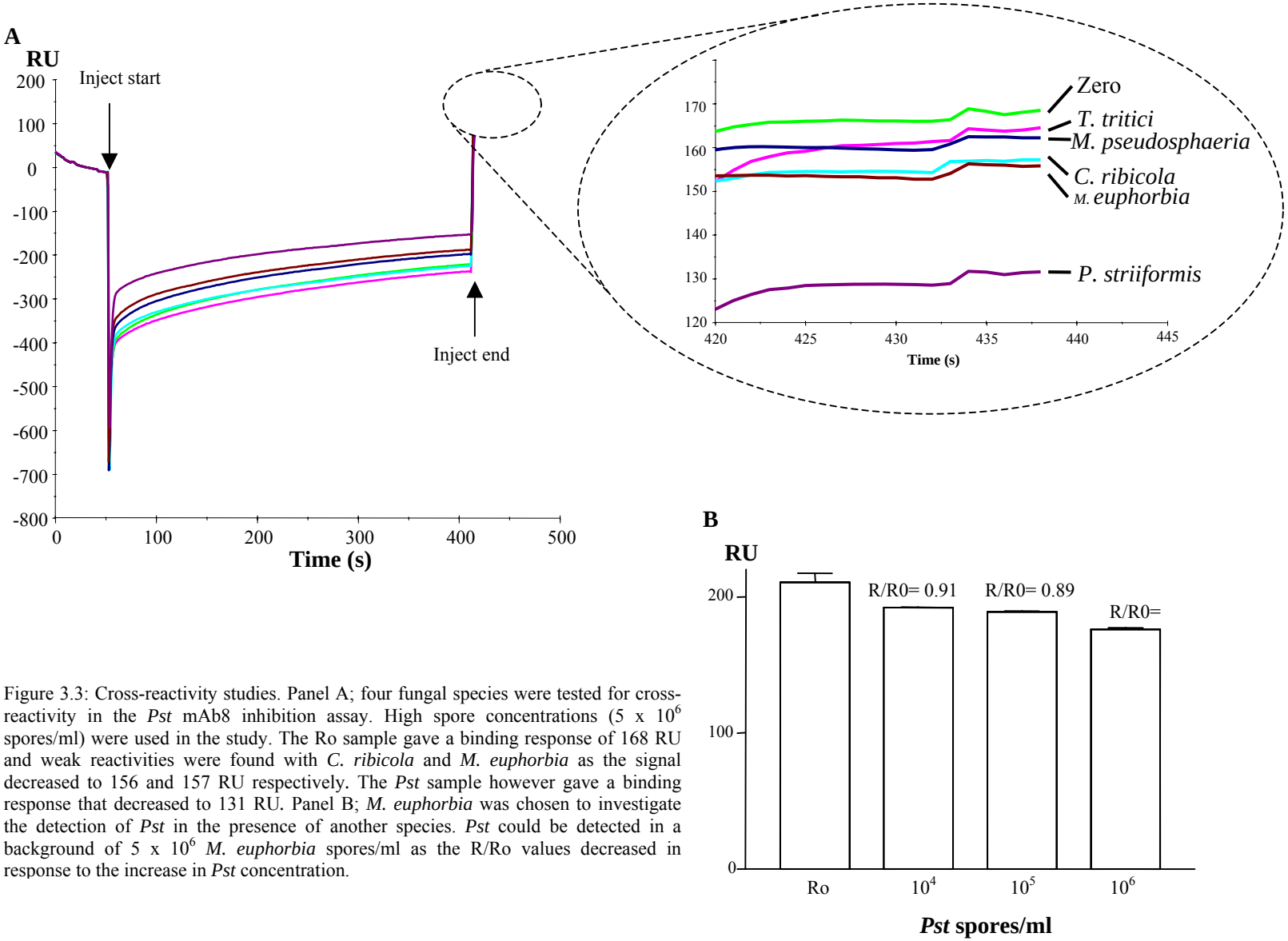

Figure 3.3: Cross-reactivity studies. Panel A; four fungal species were tested for cross-reactivity in the *Pst* mAb8 inhibition assay. High spore concentrations (5 x 10^6 spores/ml) were used in the study. The Ro sample gave a binding response of 168 RU and weak reactivities were found with *C. ribicola* and *M. euphorbia* as the signal decreased to 156 and 157 RU respectively. The *Pst* sample however gave a binding response that decreased to 131 RU. Panel B; *M. euphorbia* was chosen to investigate the detection of *Pst* in the presence of another species. *Pst* could be detected in a background of 5 x 10^6 *M. euphorbia* spores/ml as the R/Ro values decreased in response to the increase in *Pst* concentration.

The achieved detection limit of 3.1 x 10^5 urediniospores/ml was similar to the detection limits of 10^4 cfu/ml and 10^5 cfu/ml reported for *L. monocytogenes* and *Salmonella spp.* using subtractive inhibition assays (Haines and Patel, 1995; Leonard et al., 2004). The results in this study demonstrate the merits of the biosensor approach in terms of analysis time, automation, and sensitivity. Sample measurement time was approximately 45 minutes, which includes sample incubation, centrifugation and measurement of binding response. Furthermore, the use of the automatic Biacore® 3000 system reduced hands-on time, as many samples could be included in the centrifugation step and processed in overnight runs, thereby increasing sample throughput.

4. Conclusions

The generic biosensor setup described in this paper could feasibly be used for detection of any fungal spore, the only requirement being the availability of a suitable antibody. Specific antibodies have been developed against multiple fungal and fungal-like spore species. These antibodies include polyclonal antibodies, monoclonal antibodies and phage-displayed single-chain Fv antibody fragments (scFv) for important agricultural pathogens such as *Phytophthora infestans* and *Botrytis cinerea* (Bossi and Dewey, 1992; Gough et al., 1999; Werres and Steffens, 1994). By immobilising a suitable capture molecule on the sensor chip, such as an anti-isotype antibody, anti-Fab antibody or protein A/G, all available fungal pathogen specific antibodies can potentially be adapted for label-free detection using the subtractive inhibition approach described in this paper. Traditionally, *Pst* detection is performed by visual inspection of infected leaves. This requires skilled personnel and is labour intensive. Due to the simplicity of the sensor setup presented herein, untrained non-plant pathologists will be able to detect *Pst* urediniospores with high confidence. This biosensor represents the first demonstration of SPR technology for fungal spore detection and is the first biosensor for *Pst* detection reported. The assay has the potential to be implemented in future on-site sensors and current work is focused on its transfer to portable SPR sensors, which will enable us to conduct trials for detection of *Pst* urediniospores in field conditions.

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4. PAPER 3

Rapid Detection of *Phytophthora infestans* Using a Surface Plasmon Resonance Immunosensor

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ABSTRACT

Phytophthora infestans is the cause of late-blight disease and is an economically important pathogen worldwide. Early disease detection is important to implement disease control measures and in this study a Surface Plasmon Resonance (SPR) immunosensor for detection of *P. infestans* sporangia is presented. The specificity of an existing monoclonal antibody (phyt/G1470 mAb) against *P. infestans* was investigated in plate-trapped antigen ELISA and no cross-reactivity was observed against representatives from ascomycetes, deuteromycetes as well as basidiomycetes with airborne spores. phyt/G1470 mAb was incorporated in a subtractive inhibition SPR assay, consisting of a phyt/G1470 mAb and sporangia pre-incubation, a centrifugation step to remove sporangia-bound phyt/G1470 mAb and quantification of remaining phyt/G1470 mAb by SPR. Intra- and interday assay variability studies were carried out and excellent reproducibility was observed. The assay had a detection limit of 2.2 $x10^6$ sporangia/ml. Analysis was 75 minutes, which is superior to existing *P. infestans* detection methods.

1. INTRODUCTION

The fungus-like organism *Phytophthora infestans* is a major plant pathogen and the cause of late blight disease in potato and tomato plants in many parts of the world. *P. infestans* spreads by wind-dispersed sporangia produced on branched hyphae that emerge from the stomata of infected leaves under humid conditions (*1*). Detecting *P. infestans* at an early stage is crucial to minimise crop losses by implementation of correct control measures, such as crop spraying. Climate model decision support systems are used to predict favourable conditions for pathogen reproduction and disease spread (*2*). These models help farm managers in their decision to apply fungicide in a pre-emptive and rational manner. However, differences in climate and local *P. infestans* populations suggest that these methods are not always useful in geographical regions other than the one in which they were developed (*3-5*). Therefore there is an increasing demand for systems that can further aid in the early warning against *P. infestans*.

Spore traps are typically used to collect air-borne fungal spores on-site in crop fields. Pathogens in the collected samples are identified by direct microscopic identification or by *in vitro* cultivation of spores and identification of characteristic fungal structures. These methods are labour intensive, the throughput is low and analysis time can vary from several days to weeks (*6*). Current molecular detection methods for *P. infestans* are based on polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA) (*7-10*). Although these assays are very sensitive and specific, they are labour intensive, label-dependent and therefore confined to a specialised laboratory environment. An alternative flow cytometry based assay which was able to discriminate *P. infestans* from other airborne particles was recently described. The success of this approach was however dependent of sporangia labelling with fluorescent brightener and on complex data evaluation (*11*). Future on-site detection sensors should preferable monitor airsamples in a fast and continuous manner. This means that the detection should be label-free to minimise analyses time and to produce a result, which can be interpreted by personnel with limited training.

In recent years there has been a focus on development of immunobiosensors for detection of food-borne pathogenic bacteria such as *Listeria monocytogenes*, *salmonella sp.* and *Escherichia coli* (*12-15*) as well as various biowarfare pathogens (*16-18*). Although detection of microorganisms in agriculture is highly important in order to limit crop losses, there is a lack of biosensors for detection of plant pathogens.

The surface plasmon resonance (SPR) technology is an optical detection principle that is used in many commercial sensors. In SPR systems it is possible to study 'real-time' molecular interactions and the technology has been used for detection of several different analytes (for a review see (*19*)). The most widely used SPR sensor is the Biacore® system, in which the binding of analytes to immobilised ligands can be studied. The sensor measures refractive index changes on and within 300 nm from the sensor surface, and the refractive index changes are proportional to the mass changes that occur upon analyte binding. Biacore® immunosensors have been used for the detection of bacteria cells either by their binding to immobilised antibodies on sensor chip surfaces or by indirect subtractive inhibition assays (*15;20-22*). As the diameter of most cells exceeds the 300 nm range, a large number of cells are needed to produce a significant change in response units (RU) in assays with direct surface-capture (*21;23-26*).

The subtractive inhibition assay is an indirect method, consisting of a ligand-antibody interaction. This makes whole cell detection both more easy and sensitive than direct cell capture. The method is based on a pre-incubation of cells and antibody, removal of cell-bound antibody, followed by a quantification of the remaining unbound antibody by a Biacore® sensor surface capable of antibody binding. The remaining free antibody is thereby related to the initial cell concentration (*21;22*). The method is suitable for analyses of complex matrices such as food samples, homogenised plant tissue, viscous material and dirt samples, which can all potentially block the microfluidic path and damage the sensor instrument.

An SPR biosensor capable of detecting *P. infestans* is a useful supplement to early warning climate models. The wind-dispersed *P. infestans* sporangia are large, with a diameter range of 12-23 μm, thus making them unsuitable for direct injection into SPR sensors. In this study an existing *P. infestans* mouse monoclonal antibody (mAb) is used in conjunction with a subtractive inhibition assay approach to develop a label-free SPR immunobiosensor capable of sporangia detection.

2. MATERIALS AND METHODS

2.1 Spore production

A 1:1 mixture of *P. infestans* mating type A1 and A2 were used in this study. The isolates were cultivated on ecological Pea Rye agar plates. Sporangia were extracted from agar plates by PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and stored at -20 °C until further use. The following species representing ascomycetes, deuteromycetes as well as basidiomycetes with airborne spores were used for cross-reactivity studies*, Botrytis cinerea, Epicoccum sp*., *Blumeria graminis, Tilletia tritici, Melampsora euphorbia*.

2.2 *Phytophthora mAb*

A mouse monoclonal antibody (phyt/G1470) raised against *Phytophthora* mycelium was a kind gift from Agdia Incorporated. The mAb was received as an ammoniumsulphate precipitate and was further purified by proteinA affinity chromatography.

2.3 Immunofluorescence microscopy

P. infestans **s**porangia were isolated from agar plates in water and 20 µl of the spore suspension (5 x 10^5 spores/ml) were applied to multiwell glass slides and allowed to germinate for 16 hours at room temperature. The glass slides were dried in a laminar air flow hood, followed by fixing with 20 µl of 3 % paraformaldehyde for 30 minutes. Next the glass slides were incubated with 20 µl 1 % (w/v) skimmed milk powder (Sigma-Aldrich, St Louis, USA) in PBS for 30 minutes. Three-fold dilution series of phyt/G1470 mAb in PBS-T (PBS containing 0.1 % (v/v) Tween20) were made in the range 0.03 – 8 µg/ml. Twenty µl of each phyt/G1470 mAb solution was applied to each well and incubated for 60 minutes. A 2.7 µg/ml phyt/G1470 mAb solution was found to give the best fluorescence intensity with limited background fluorescence. Next 20 µl FITC (fluorescein-5-isothiocyanate)-conjugated goat anti-mouse IgG immunoglobulin (Sigma-Aldrich) diluted 1:64 in PBS-T was added and incubated for 60 minutes. Following each step the glass slides were washed 3 times 5 minutes in PBS-T. The glass slides were dried and one drop of CITIFLUOR (AGAR Scientific Limited, Essex, England) was added to each well and a coverslip was applied covering all wells. The fluorescence was observed with an UV Leitz Laborlux S microscope and

documented using a camera connected to the microscope. Control wells were included, in which phyt/G1470 mAb was replaced with PBS-T, but otherwise treated similar.

2.4 Cross-reactivity studies by plate-trapped antigen ELISA (PTA-ELISA)

100 ul of each spore species suspension (5 x 10^5 spores/ml) in PBS was added to each well in MaxiSorp™ plates (Nunc Glostrup, Denmark) and incubated for 16 hours at 37 °C, followed by blocking with 200 µl 1 % (w/v) skimmed milk powder in PBS. Wells were washed with PBS-T and 100 µl phyt/G1470 mAb (diluted to 1 µg/ml in PBS-T) was added. Alkaline phosphatase (AP)-conjugated polyclonal goat anti-mouse IgG+IgM (Immunokontakt, Wiesbaden, Germany) diluted 1/2500 in PBS-T was added to PBS-T-washed wells. The ELISA was developed using 100 µl/well of pNPP AP-substrate (Sigma-Aldrich) and incubated for 30 minutes in the dark at 37 °C. Each spore species was analysed in triplicate. Unless otherwise stated all steps were performed for one hour at 37 °C.

2.5 Subtractive inhibition ELISA

Optimal assay conditions were identified by checkerboard ELISA. Microtitre plates were coated with 0.2 µg/well polyclonal rabbit anti-mouse IgG1 (SouthernBiotech, Birmingham, UK) in 100 µl PBS for 1 hour at 37 °C. The coated wells were washed with PBS and blocked with 200 µl 1 % (w/v) skimmed milk powder in PBS. Sporangia standards (200 µl of each concentration) were prepared by a three-fold dilution series in HBS (10 mM HEPES, 150mM NaCl, 3mM EDTA, 0.2% (v/v) Tween, pH 7.4) and phyt/G1470 mAb (200 µl of 0.1 µg/ml) was added yielding a final 0.05 µg/ml phyt/G1470 mAb concentration. The mixture was incubated for 60 minutes at 37 °C and inverted frequently to allow good mixing of spores and phyt/G1470 mAb. Sporangia-bound phyt/G1470 mAb were removed from the remaining free phyt/G1470 mAb by centrifugation at 1500 x g for 5 minutes. The phyt/G1470 mAb-containing supernatants were carefully removed and added to ELISA plates in quadruple wells (100 µl/well) for each spore concentration. AP-conjugated goat anti-mouse IgG+IgM (Immunokontakt) diluted 1/2500 in PBS-T was added to the wells. Plates were washed with PBS-T and 100 µl of pNPP AP-substrate was added before incubation for 30 minutes in the dark at 37 °C. The absorbance was read at 405 nm. Unless otherwise stated all incubation

steps were performed for one hour at 37 °C. The average response from the quadruple measurements (A) was divided by the average response of phyt/G1470 mAb in HBS only (Ao) to give normalised values. Cross-reactivity studies were performed with serial dilutions of inhibiting antigen (*B. cinerea, Epicoccum sp*., *B. graminis, T. tritici, M. euphorbia*) alongside the same dilutions with *P. infestans* sporangia. Percent cross-reactivity (CR) were estimated using the expression; CR = (C/C*) x 100 %, where C and C* are the IC_{10}-values for *P. infestans* and the reference spore, respectively.

2.6 Development of a Biacore®-based subtraction inhibition assay

2.6.1 Antibody immobilisation

Analyses were performed with a Biacore® 3000 sensor (Biacore®, Uppsala, Sweden) using filtered and degassed HBS running buffer and a CM5 dextran surface (Biacore®). The optimal pH for immobilisation (preconcentration) of commercial goat anti-mouse IgG1 polyclonal antibody (SouthernBiotech, Birmingham, UK) was determined by diluting the antibody (10 µg/ml) in 10 mM sodium acetate buffers with pH from 4 to 5.5. Each buffer was injected (10 µl, flowrate 10 µl/min) over a blank flow cell in a CM5 chip and pH 4.5 was found to be optimal for immobilisation. The chip was activated by a 70 µl injection of a mixture of 50 µl of 100 mM N-hydroxysuccinimide (NHS) with 50 µl of 400 mM N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) at a flowrate of 10 µl/min. Goat anti-mouse IgG1 was immobilised by injection of 100 µg/ml diluted in 10 mM sodium acetate, pH 4.5 at a flowrate of 10 µl/min over the activated dextran surface for 10 minutes. Unreacted sites were subsequently deactivated by injecting 1 M ethanolamine, pH 8.5 (Biacore®) at 10 µl/min for 7 min.

2.6.2 Surface regeneration

The surface was effectively regenerated using 30 µl glycine-HCl, pH 2.0 at a flowrate of 10 µl/min.

Surface regeneration and stability was investigated by repeated injections of 10 µl 2.5 µg/ml phyt/G1470 mAb in HBS buffer at a flowrate of 5 µl/min followed by regeneration as above.

2.6.3 Assay setup

Sporangia concentration standards were made by serial dilutions in PBS (75 µl samples). 75 µl of phyt/G1470 mAb (5 µg/ml) in HBS buffer was added to each solution, giving a final mAb dilution of 2.5 µg/ml. The mixtures were incubated and centrifuged as described for the subtractive inhibition ELISA. 100 ul of each supernatant were carefully removed without touching the spore pellet. 10 µl of each supernatant was assayed randomly in duplicate by automated analysis using the Biacore® 3000 instrument. The samples were injected over the CM5 chip surface at a flow rate of 5 µl/min and the resulting response unit change was measured at the end of each injection. Following each sample injection the surface was regenerated as described above. The assay was repeated on three separate days and three times on the same day to generate interday and intraday variation data respectively. Mean values from duplicate measurements (R) and a blank sample response containing only phyt/G1470 mAb (R_0) was calculated and normalised values (R/Ro) were obtained, thereby obtaining the degree of inhibition for each spore concentration. A calibration curve was constructed by fitting the data to a four-parameter logistic function using the Bia-evaluation software (version 4.0.1). The detection limit was calculated as the lowest spore concentration resulting in 10 % inhibition (IC_{10}) as suggested previously (*27*).

3. RESULTS

3.1 phyt/G1470 mAb sporangia binding

The mouse phyt/G1470 mAb used in this study was raised against *Phytophthora* mycelium. To verify that the phyt/G1470 epitope for the mAb was present on sporangia as well as mycelium structures, immunofluorescense microscopy was performed using sporangia that had germinated on glass surfaces. Fluorescense was observed from the sporangia and in the protruding germ tubes (figure 4.1, panel A). This fluorescence was not seen in the control experiment (figure 4.1 panel C) and illustrates the suitability of phyt/G1470 mAb for *Phytophthora* sporangia detection.

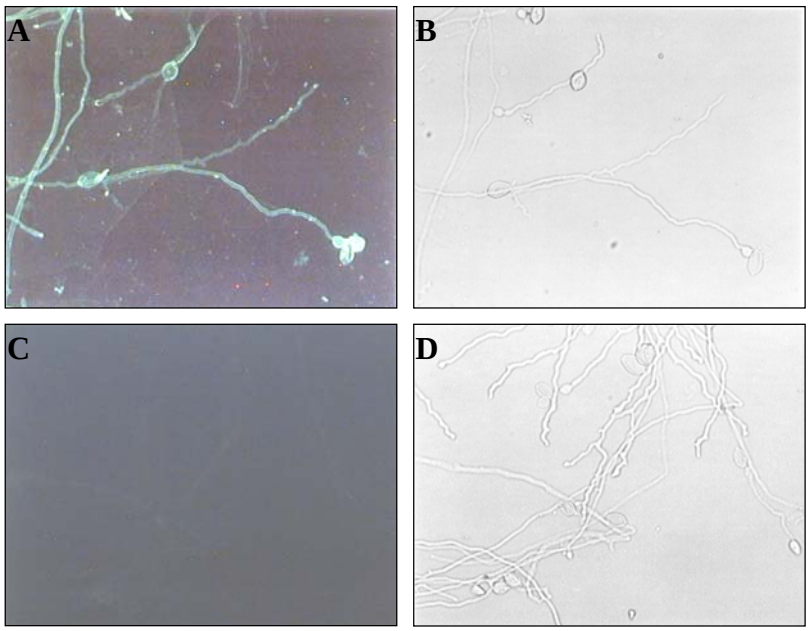

Figure 4.1: Immunofluorescence microscopy using phyt/G1470 mAb. Germinated *P. infestans* sporangia was used as the antigen. Panel A shows the binding of phyt/G1470 mAb to sporangia and germ tubes under UV light. Panel B: the same as A, but viewed in normal light. Panel C: control well with phyt/G1470 omitted. Panel D: same as C but viewed in normal light. Fluorescence is observed from the sporangia and the protruding germ tubes. The control experiment is lacking fluorescence, thereby confirming the binding of phyt/G1470 mAb to sporangia.

3.2 phyt/G1470 mAb cross-reactivity profile

The phyt/G1470 mAb is the detection antibody in a commercial Double Antibody Sandwich ELISA (DAS-ELISA), which detects *Phytophthora* to the genus level only. No cross-reactivity has been observed in the DAS-ELISA against isolates of *Aspergillus sp., Fusarium sp., Monilinia sp., Penicillium sp., Rhizoctonia sp.,*

Sclerotinia sp. and some *Pythium sp* (www.agdia.com). However, weak to moderate cross-reactivity has been observed against 10 *Pythium* species (www.agdia.com). To further expand the phyt/G1470 mAb cross-reactivity profile, additional isolates representing genera with airborne spores were tested by PTA-ELISA. The reactivity towards *B. cinerea, B. graminis, Epicoccum sp*., *M. euphorbia and T. tritici* were found to correspond to that of the control background (PBS coat only), illustrating a high specificity of phyt/G1470 (figure 4.2 panel A).

3.3 Evaluation of a subtractive inhibition assay

A subtractive inhibition assay (incorporating phyt/G1470) was developed for *P. infestans* detection. Fungal spores from *B. cinerea, B. graminis, Epicoccum sp*., *M. euphorbia* and *T. tritici* were analysed alongside *P. infestans* to investigate the cross-reactivity profile of phyt/G1470 in the subtractive inhibition assay (figure 4.2 panel B). From the lowest spore concentration resulting in >10% inhibition, IC_{10}-values could be obtained and the percentage-cross-reactivity estimated for each spore species. Weak cross-reactivity (~ 11 %) was found with four species (*T. tritici, Epicoccum sp., B. cinerea* and *B. graminis*) but based on the PTA-ELISA results it is concluded that the reactivity is due to unspecific reaction of phyt/G1470 mAb with increasing spore concentrations. This supports the subtractive inhibition assay in terms of specificity.

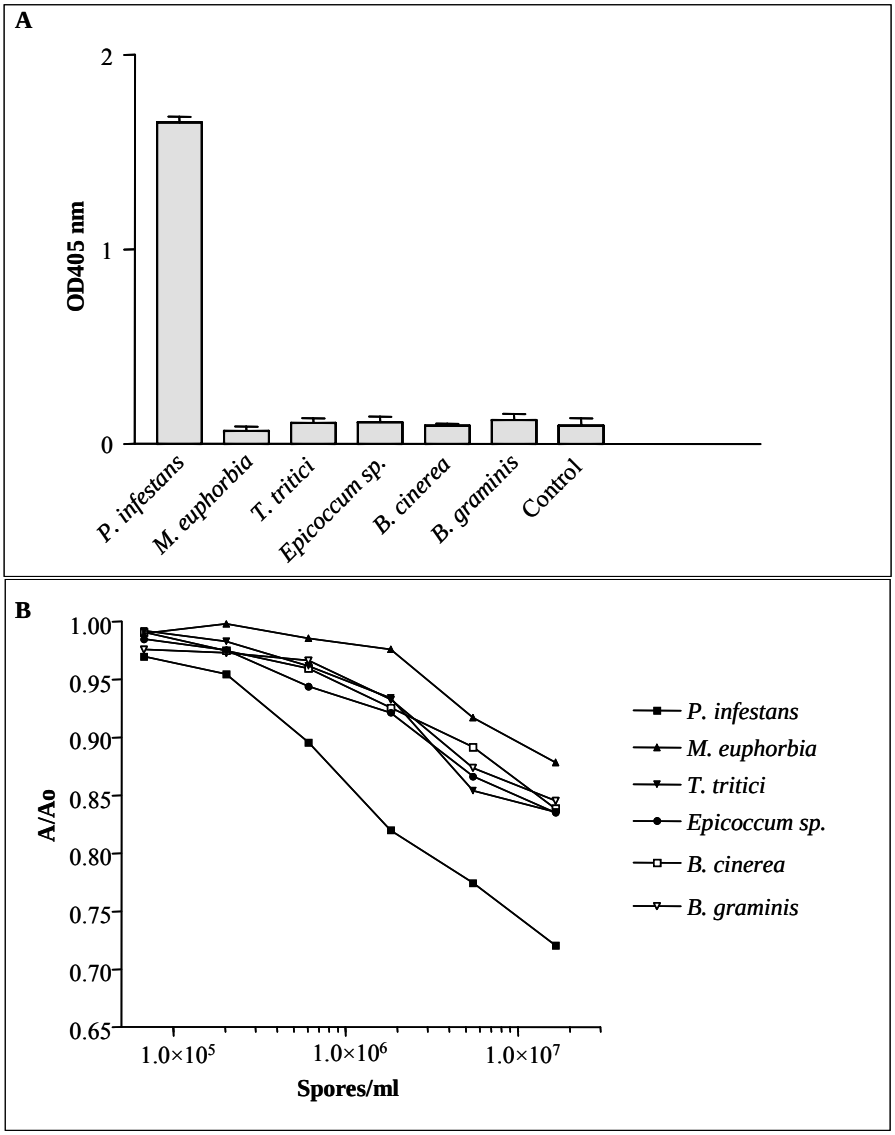

Figure 4.2: Cross-reactivity of phyt/G1470 mAb. Panel A: The antibody was tested against seven genera representatives (*B. cinerea, B. graminis, C. ribicola*, *Epicoccum sp*., *M. euphorbia, M. pseudosphaeria and T. tritici*) by PTA-ELISA. Reactivity of phyt/G1470 mAb was only found in *P. infestans* sporangia coated wells. Results shown are mean and standard deviations from triplicate measurements. Panel B: Cross-reactivity of phyt/G1470 mAb in a subtractive inhibition assay. Different spore species (*B. cinerea, B. graminis, Epicoccum sp*., *M. euphorbia and T. tritici*) were analysed alongside *P. infestans* sporangia. Normalised values (A/Ao) were plotted against the spore concentration. IC_{10}-values were obtained and the percentage-cross-reactivity estimated for each spore species. The following percent cross-reactivities were estimated; *M. euphorbia* (3.7 %), *T. tritici* (11.1 %), *Epicoccum sp*. (11.1 %), *B. cinerea* (11.1 %), *B. graminis* (11.1 %).

3.4 SPR assay preparation

The subtractive inhibition assay was further implemented into a Biacore® 3000 SPR sensor. A goat anti-mouse IgG1 polyclonal antibody was immobilised to a CM5 chip surface (approximately 17000 RU, data not shown). A 2.5 µg/ml solution of phyt/G1470 mAb gave an approximate binding response of 250 RU. The binding response was compared to that of a blank chip surface, which gave insignificant binding, thereby illustrating the specificity of the binding response (data not shown). Optimal regeneration conditions were investigated and a 30 µl pulse of 10 mM glycine-HCl, pH 2.0 at a flowrate of 10 µl/min was shown to be sufficient for regeneration (figure 4.3 panel A). To investigate long-term surface stability, repeated antibody binding and regeneration cycles were performed (figure 4.3 panel B). This demonstrated that the surface could be regenerated more than 100 times, with only a 6 % decrease in the surface activity from the first to the last binding cycle. The chip surface was used throughout the analyses described in this paper and the surface activity did not decrease below 20%.

3.5 SPR assay setup

The subtractive inhibition assay was performed by incubating decreasing concentrations of sporangia with phyt/G1470 mAb. The spore-bound phyt/G1470 mAb was removed by centrifugation and the remaining unbound mAb was subsequently quantified using the goat anti-mouse IgG1 CM5 surface and the Biacore® 3000 instrument. As seen from the overlay plot in figure 4.4, a binding response inversely proportional to the spore concentration was obtained, thereby verifying the biosensing principle. Sporangia standards were made and analysed randomly in duplicate alongside a zero sample containing phyt/G1470 mAb only. The average response (R) from each spore concentration was calculated and divided by the Ro average response to obtain normalised values (R/Ro) for each sample set. The normalised values were fitted to a four-parameter logistic function to construct a calibration curve showing correlation between degree of inhibition and sporangia concentration (figure 4.5). The range of detection was found to be approximately 1.39×10^5 - 3.55×10^7 sporangia/ml and based on the IC_{10}-value a detection limit of 2.22×10^6 sporangia/ml was achieved.

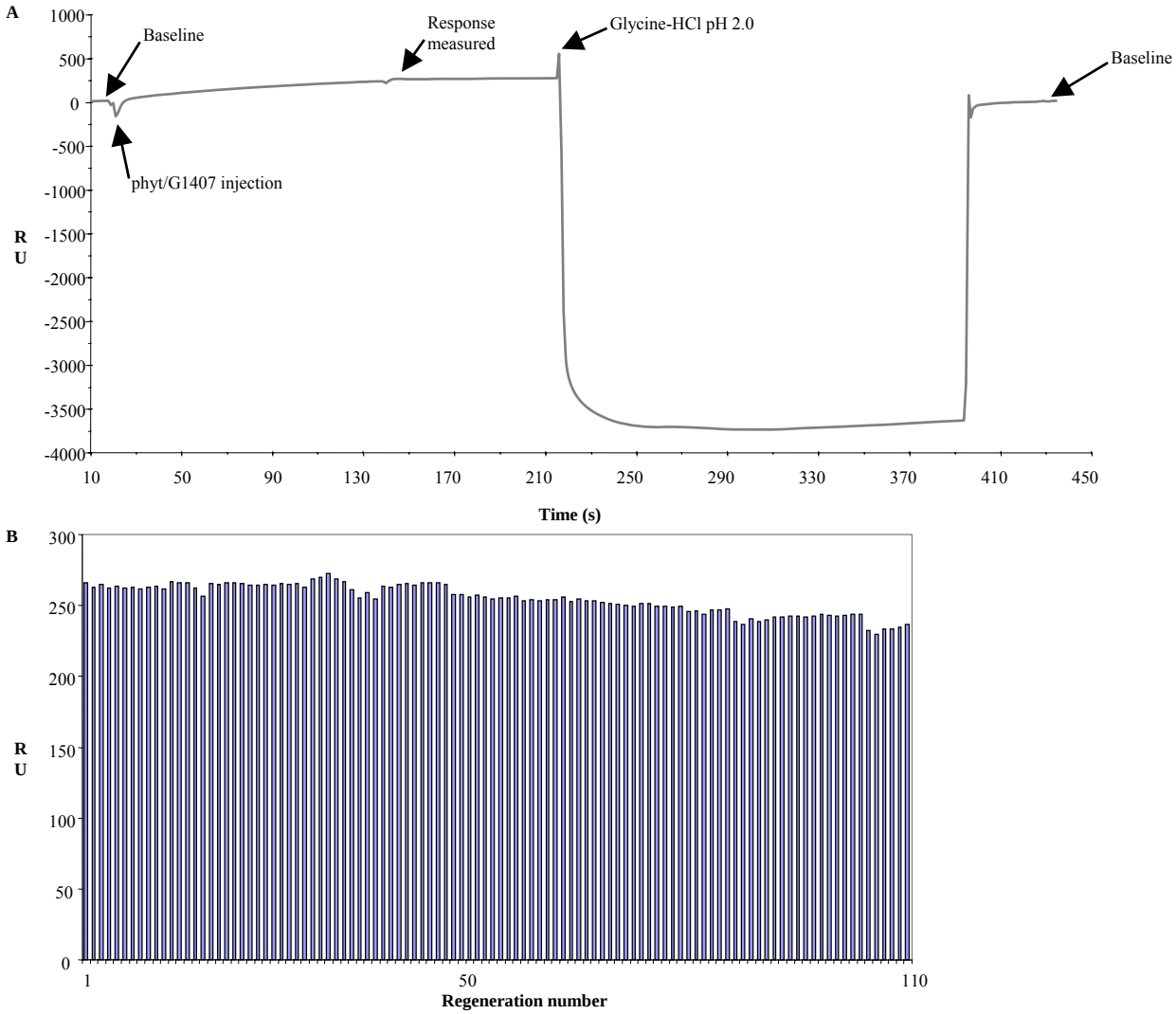

Figure 4.3: Regeneration and surface stability. Panel A shows a complete binding and regeneration cycle using a 2.5 µg/ml protein A purified phyt/G1470 mAb in HBS, and glycine-HCl pH 2.0. After phyt/G1470 mAb injection the resulting response is measured and following regeneration the baseline response is restored. Panel B shows the surface stability in 110 binding and regeneration cycles. A decrease in surface activity of 6 % from the first binding cycle (251.5 RU) to the last (236.4 RU) was observed.

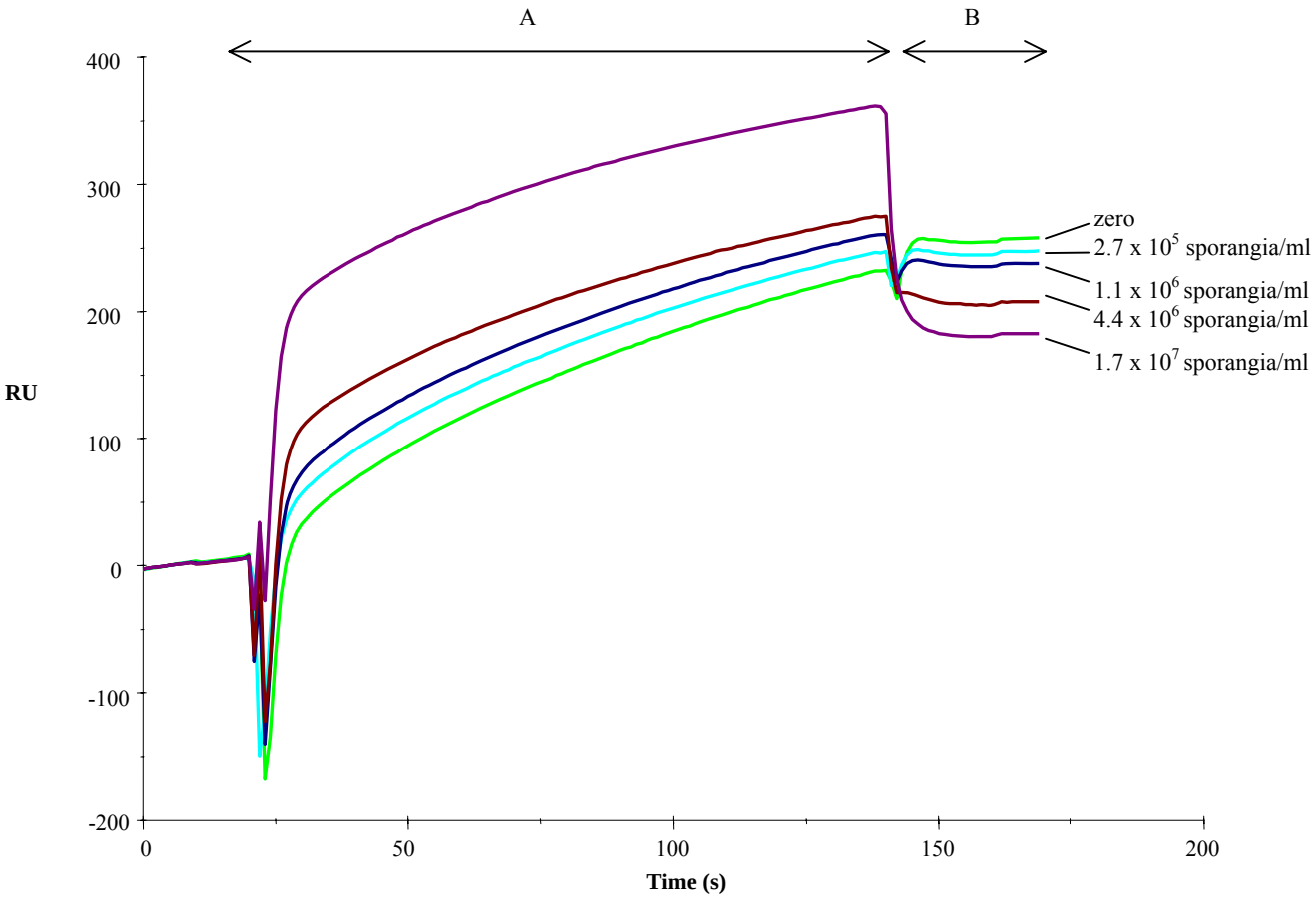

Figure 4.4: Overlay plot demonstrating the binding response, being inversely proportional to the sporangia concentration. Representative results from sporangia concentrations ranging from 2.7 x 10^5- 1.7 x 10^7 sporangia/ml are shown. Section A of the sensorgram is the injection phase and section B is the washing phase where the final response units are measured.

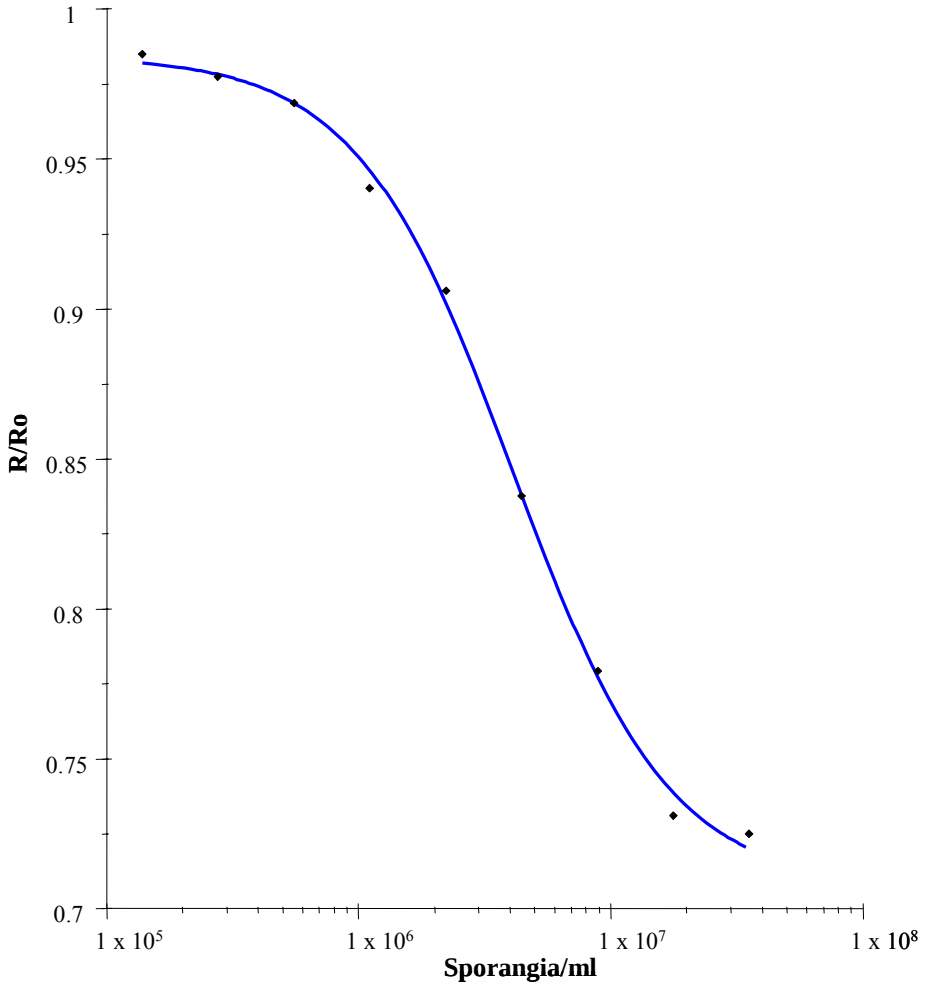

Figure 4.5: Interday calibration curve. *P. infestans* sporangia standards in the range 1.3 x 10^5 - 3.5 x 10^7 were prepared and analysed in triplicate on 3 different days. The normalised responses (R/Ro) were plotted against the sporangia concentration to illustrate the degree of inhibition. From the IC_{10} value a detection limit of 2.22 x 10^6 sporangia/ml was achieved.

3.6 SPR assay performance

The assay stability was investigated by interday and intraday variability studies. The coefficients of variation (CV's) for each point of the standard curves was found to be in the range from 0.09-5.47% and 0.48-4.83 for the interday and intraday studies respectively. This illustrated good reproducibility of the immunoassay (table 4.1). The percentage accuracy between the fitted four-parameter logistic function and the actual concentration used was determined and the data fitted very well to the curves with percent-accuracies ranging from 80.18-128.6 % in the interday analysis and 72.07-142.15 % for the intraday analysis. This indicates that the four-parameter logistic function is an appropriate model to describe the immunoassay.

	Interday			Intraday		
Concentration	Back-calculated conc.	CV's	%	Back-calculated conc.	CV's	%
(sporangia/ml)	(cells/ml)	%	Accuracies	(cells/ml)	%	Accuracies
3.55×10^7	2.76×10^7	5.47	128.6	$3{,}11 \times 10^7$	2.81	87.60
1.78×10^7	2.22×10^7	2.84	80.18	$1{,}72 \times 10^7$	2.01	96.98
8.88×10^6	8.62×10^6	3.27	103.02	$9{,}95 \times 10^6$	1.60	112.05
4.44×10^6	4.45×10^6	3.03	99.78	$4{,}40 \times 10^6$	3.85	99.12
2.22×10^6	2.11×10^6	3.00	105.21	$1{,}99 \times 10^6$	4.83	89.76
1.11×10^6	1.25×10^6	1.48	88.8	$1{,}19 \times 10^6$	2.80	107.05
5.55×10^5	5.51×10^5	1.64	100.73	$7{,}88 \times 10^5$	1.96	142,15
2.77×10^5	2.99×10^5	0.79	92.64	$2{,}00 \times 10^5$	1.09	72,07
1.39×10^5	-	0.09	-		0.48	-

Table 4.1: Coefficients of variation (CV's) and percent accuracies from interday and intraday studies. Interday % CV's (% CV= (standard deviation/mean) x 100 %) were obtained from 3 assays performed on three different days, while the intraday CV's were from 3 assays performed on the same day. The percent accuracy was calculated using the expression ((actual sporangia concentration/back-calculated sporangia concentration) x 100%). (*) refers to that no data could be obtained because the data point did not fit the calibration curve.

4. DISCUSSION

In this study phyt/G1470 mAb is used in a subtractive inhibition assay for *P. infestans* detection. The assay was characterised in terms of cross-reactivity and evaluated in a label-free SPR immunosensor format.

Several mAbs have been found to target epitopes that are confined to specific fungal structures. This have resulted in the production of several mAbs that detects mycelium but not spores from the same species (*28;29*). phyt/G1470 mAb was raised against mycelium but by immunofluorescence microscopy it was found that phyt/G1470 mAb did target an epitope present in both mycelium and sporangia and was therefore found suitable for sporangia detection. phyt/G1470 mAb has been reported to be highly specific for species of the *Phytophthora* genera when used in the DAS-ELISA from Agdia (www.agdia.com). It is a common finding that mAbs are only specific to the genus level only (*30*), however some cross-reactivity has also been observed for phyt/G1470 mAb towards some *Pythium* species. This indicates a shared epitope for phyt/G1470 mAb within *Phytophthora* and *Pythium* species and could be due to the close taxonomic relationship between the Omycetes *Phytophthora* and *Pythium*.

To expand cross-reactivity studies of phyt/G1470 mAb, PTA-ELISA using spores from five additional representative fungal genera was performed. No cross-reactivity for phyt/G1470 mAb towards these species was found, further illustrating the high specificity of the mAb. phyt/G1470 mAb was incorporated in a subtractive inhibition ELISA for characterisation of the cross-reactivity profile of the assay setup. Limited cross-reactivity of the assay was found and from IC_{10}-values percentage-cross reactivity was found ranging from 4 - 11 %. However, based on the PTA-ELISA results this cross-reactivity probably does not indicate a shared epitope for phyt/G1470 mAb in these species, but rather an unspecific binding of phyt/G1470 mAb to the increasing spore concentrations. Based on this knowledge, the specificity of phyt/G1470 mAb for *P. infestans* in the subtractive inhibition assay was underlined. However, as phyt/G1470 mAb display affinity for other members of the *Phytophthora* genera (www.agdia.com), the subtractive inhibition assay is probably not exclusively specific for *P. infestans*, but further investigations will have to be performed in order to fully establish this.

Current *P. infestans* detection assays have analyses times from approximately four hours (ELISA) up to several days (PCR) (*7-10*). The subtractive inhibition ELISA for *P. infestans* detection have an analysis time similar to that of existing ELISA's. To decrease analysis time, the subtractive inhibition assay was implemented in a Biacore® 3000 sensor, which is fully automated, has precision liquid handling and integrated microfluidics. This allows for rapid and precise analyses of multiple samples, thereby decreasing analysis time and reducing the hands-on-time. In addition Biacore® assays have repeated analysis on the same functionalised surface, which reduces the assay price, if suitable regeneration conditions can be identified. The anti-mouse IgG1 antibody was immobilised on a CM5 chip and suitable regeneration conditions identified. A single injection of 10 mM glycine-HCl, pH 2.0 was sufficient to remove phyt/G1470 mAb from the chip surface. The effect of low pH buffers are believed to be due to a reversible partial unfolding of the ligand and analyte making them positively charged, resulting in molecule repulsion as the binding sites no longer match (*31*). During regeneration care should be taken not to effect the activity of the ligand, as the lifetime of the sensor surface can be compromised. Excellent longterm surface performance was found by repeated phyt/G1470 mAb binding and regeneration cycles, as 110 regenerations resulted in a 6 % surface activity decrease. During the course of the study the surface activity did not decrease more than 20 %, which is the recommended cut-off value that ensures validity in Biacore® assays with repeated surface regenerations (*32*).

The association phase on the overlay plot (section A on figure 4), surprisingly shows binding responses for sporangia containing samples that are proportional to the spore concentration. However, following the shift to HBS running buffer, a binding response that is inversely proportional to the sporangia concentration is seen (figure 4.4, section B). The SPR instrument measures refractive index changes both on and near the sensor surface. This means that the sporangia concentration-dependent response in the injection phase is due to soluble sporangia surface components passing within close proximity to the sensor surface. However when the shift to HBS running buffer occurs (figure 4.4, section B) only the specific binding of phyt/G1470 mAb is observed, thereby confirming the high specificity of the sensor surface.

The assay had a detection range from 1.39 x 10^5 - 3.55 x 10^7 sporangia/ml and based on the IC_{10}-value a detection limit of 2.22 x 10^6 sporangia/ml was achieved (the R/Ro-value at this concentration was 0.906). Looking at the linear part of the curve (figure 4.5) it is evident that the dynamic range of the assay is narrow. Furthermore, R/Ro-values only decreases to approximately 0.70, which is surprising as the R/Ro-values would be expected to decrease as the sporangia concentration increases. This effect was also observed in subtractive inhibition ELISA's suggesting that the phenomenon is phyt/G1470 mAb-dependent A plausible explanation is that at high sporangia concentrations the cells stick together, thereby making epitopes inaccessible to phyt/G1470 mAb. It is likely that improvements of the assay performance can be achieved using other available *P. infestans* antibodies (*10*). However, as phyt/G1470 mAb was found to be highly specific, changing the antibody can comprimise the high specificity of the assay.

4. CONCLUSIONS

The data presented in this study represents the first biosensor-based approach for *P. infestans* detection. The biosensor is currently confined to a laboratory environment but future work is focused on the transfer of the assay onto portable sensors, where the centrifugation step will most likely be substituted by a micro-filter separation step of sporangia-bound phyt/G1470 mAb. The analysis time of the SPR assay is superior to available *P. infestans* detection assays, with a toal analysis time of approximately 75 minutes. In addition, the use of the automatic Biacore® 3000 system increases sample throughput as many samples can be processed in overnight runs.

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5. GENERAL DISCUSSION AND CONCLUSIONS

The purpose of this thesis has been to investigate the potential of Surface Plasmon Resonance (SPR) immunosensors for detection of plant pathogens. Assays were developed for detection of *P. striiformis* urediniospores and *P. infestans* sporangia using a subtractive inhibition approach. In this chapter the results of the papers are summarised and discussed. Furthermore the potential for future implementation of the assays on-site will be explored.

As no immunological reagents existed for the model organism *P. striiformis*, focus was put on the production of mAbs for detection (paper 1). Mice were immunised with urediniospores as future sensors should be able to detect intact spores. To limit the risk of selecting mAbs reacting with released intracellular components, the screening was performed using microtiter plates coated with intact urediniospores. In this way, mAbs were selected for native surface structures. The cross-reactivity profile of the mAbs was investigated and two mAbs (mAb4 and mAb8) were selected and used to develop a competitive ELISA (using mAb4) and a subtractive inhibition ELISA (using mAb8). The subtractive inhibition assay was found to be more sensitive with a detection limit of 1.5 x 10^5 urediniospores/ml. The assay was further evaluated against other rust species and cross-reactivity was found with *Puccinia recondita* and *Puccinia hordei*, suggesting that mAb8 is specific to the *Puccinia* genus level only. mAb8 targeted a ~ 39 kDa spore surface protein and based on the cross-reactivity with other *Puccinia* species, the protein could be a conserved structural component in the surface of *Puccinia* species.

A SPR immunosensor for *P. striiformis* was further developed (paper 2). This assay was based on the subtractive inhibition method and utilised the specificity of mAb8 (IgM isotype). A polyclonal anti-mouse IgM was immoblised on a flow cell in a CM5 sensor chip by amine-coupling at pH 4.5. The immobilised antibody was used for capture and quantification of mAb8. Optimal regeneration conditions were identified and 20 mM HCl effectively regenerated the surface and this solution was used for multiple regeneration cycles during the assay. From three independent assays a calibration curve was generated and a detection

limit of 3.1 x 10^5 urediniospores/ml was achieved. The ability to detect *P. striiformis* in the presence of the rust species *M. euphorbia* was tested. Using a 5 x 10^6 spores/ml of background of *M. euphorbia* it was possible to detect differences in samples spiked with 10^4, 10^5 or 10^6 *P. striiformis* urediniospores/ml. This underlined the stability of the assay in terms of specificity and the potential for the assay for measuring "real-life" samples.

For comparison, mannan-binding protein (MBP) was also investigated as a specific mAb8 capture ligand. MBP binds carbohydrate residues, which make up ~ 12 % of the pentameric mouse IgM mass. MBP affinity chromatography is a well established technique for IgM purification (*64*). During ELISA tests it was found that MBP displayed mAb8 binding capacity. Following immobilisation of MBP to a CM5 flow cell, the mAb8 binding-capability was however lost, likely due to denaturation of MBP during immobilisation (data not shown in the thesis). Consequently, this approach was not pursued any further.

A SPR immunoassay was further developed for detection of sporangia from *Phytophthora infestans* using an existing *Phytophthora* mAb (paper 3). The phyt/G1470 mAb has been found to be specific for the *Phytophthora* genera, although reactivity is detected with some species of *Pythium*. A subtractive inhibition ELISA was developed using phyt/G1470 mAb (IgG1-isotype). phyt/G1470 mAb was quantified using microtiter wells coated with polyclonal anti-mouse IgG1. The assay was evaluated for cross-reactivity against spores from species representing ascomycetes, deuteromycetes and basidiomycetes. Largely no cross-reactivity was found (max 11 %) for the species. The subtractive inhibition assay was incorporated in a SPR-assay. Optimal immobilisation conditions for the polyclonal anti-mouse IgG1 were identified and the surface could be effective regenerated using glycine-HCl pH 2.0. Calibration curves were generated by data-fitting and based on good %-accuracies, the four-parameter equation was found to be suitable to describe the immunoassay. A detection limit of 2.22 x 10^6 sporangia/ml was achieved and the analysis time of 75 minutes is superior to existing immuno- and nucleotide-based assays for *P. infestans* detection.

The sensitivity of the *P. striiformis* SPR assay (3.1 x 10^5 urediniospores/ml) was about ten-fold higher than the *P. infestans* SPR assay (2.22 x 10^6 sporangia/ml). Both assays had similar analyses times, although *P. infestans* sporangia were incubated for 60 minutes to enhance inhibition (compared to 30 minutes for the *P. striiformis* assay). The likely reason for the sensitivity difference is the antigen density on the pathogen surface. mAb8 has probably targeted an abundant antigen that is easily accessible, whereas the phyt/G1470 mAb antigen is likely not present in high amounts. The inability to reach more than approximately 30 % inhibition in the *P. infestans* assay could also suggest that the phyt/G1470 mAb antigen is buried deeper in the sporangia surface and that increasing sporangia concentrations leads to shielding of the antigen as sporangia stick together. However the performance of the *P. infestans* assay could likely be improved by using another mAb, provided that the mAb-specificity is as high as phyt/G1470 mAb.

The results described in this thesis have shown that it is possible to use SPR-technology for detection of large plant pathogens. The flow cell dimensions in Biacore® 3000 sensors are *length* = 2.4 mm, *width* = 0.5 mm and *height* = 0.025 mm (personal communication, Biacore® technical department). Provided that cells do not stick together, it is therefore theoretically possible to inject large particles up to 25 µm in diameter. This means, that bacterial cells (typically 1 - 5 µm in diameter) can easily be injected into sensors without the risk of damaging the microfluidic system. However, fungal spores typically have larger diameters (e.g. *Puccinia striformis* urediniospores are up to 45 µm), meaning that direct injection of these organisms are likely to block the microfluidic path and damage the sensor. Therefore, subtraction inhibition assays are particular suitable for analysis of these large cells.

At present, the described SPR-assays are laboratory-based due to the relatively large size of Biacore® sensors. Alternative SPR-sensors however exist that has the capability to perform analyses on-site. Recently the Spreeta™ evaluation module has been aquired at the Danish Institure of Agricultural Sciences for the purpose of analysing "real-life" samples on-site. An overview of the Spreeta™ system is seen in figure 5.1 A-D. The sensors are small and consists of a gold surface for ligand immobilisation, a source for optical interrogation and a diode array, which measures the SPR-angle (figure 5.1 A and B). Three parallel channels

for analyses can be obtained using the black flow-cell pressing against the gold surface, thereby creating the channels (figure 5.1 C). The sensor is placed in the blue flow-block which secures the flow-cell to the sensor and collects data (figure 5.1 D). The data is transferred to a PC via a caple connected to the flow-block. Three inlet and outlet tubings are furthermore connected to the flow-cell and an external pump drives the system (figure 5.1 D, pump not shown).

To accommodate a subtractive inhibition assay on-site I propose to construct the following sample analyses-model (figure 5.2). The pre-incubation step is performed in two parallel chambers, each containing the same concentration of pathogen-specific antibody (in this case a mouse IgG mAb for simplification). Spores are sampled from the air and solubilised by adding buffer (not seen on figure 5.2).

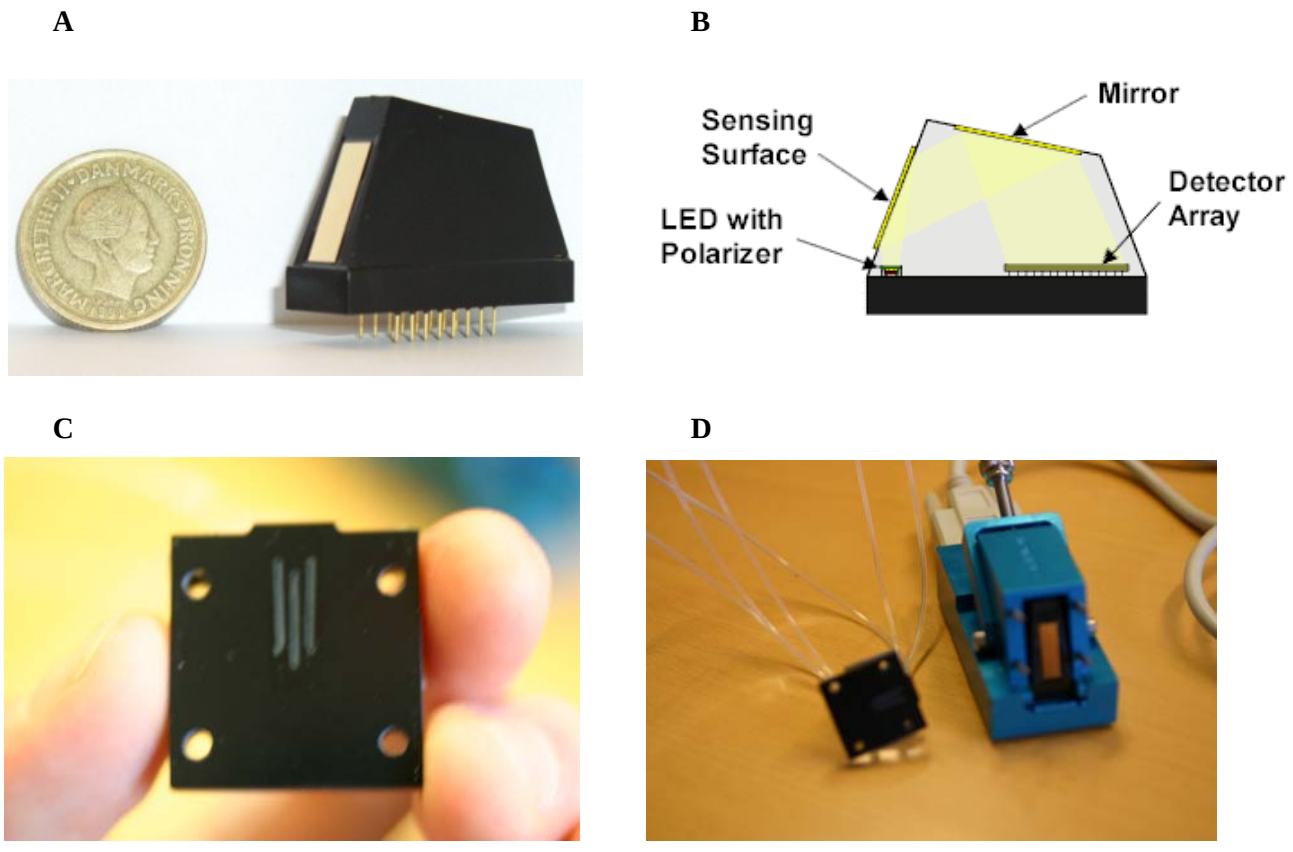

Figure 5.1: An overview of the Spreeta™ SPR sensor. **Panel A and B** shows the sensor consisting of a light source, a gold surface for ligand immobilisation and a diode array detecting the changes in the SPR-angle. **Panel C** shows the flow-cell, which can create three parallel channels for analyses when it is pressed against the sensor gold surface. **Panel D** shows a sensor placed in the blue flow-block. The flow-block transmits data to a PC via a cable. The inlet and outlet tubings connected to the flow-cell are also shown.

The spore sample is transferred into chamber1 and an equal amount of buffer is transferred into chamber2. Spores and antibody are allowed to react for a suitable amount of time, whereafter both spore-containing sample and control sample are pressed through filters. The pathogens are withheld by the filter and the antibody-containing filtrate is then pumped to the Spreeta™ sensor for analyses. The Spreeta™ sensor surface is functionalised with a polyclonal anti-mouse IgG capable of mouse IgG quantification. As illustrated on the sensor read-out, the pathogen-containing sample (red) gives a lower response compared to the control sample (blue) indicating presence of the pathogen in the collected sample.

The use of a filtration step in the proposed model means, that larger sample volumes must be used for analyses, as filters consume some of the sample. The filtration step is however more suitable for on-site analyses than the centrifugation step described in papers 2 and 3. If IgG isotype mAbs can be obtained for a broad range of plant pathogens, then the described model can accommodate the detection of many different species using the polyclonal anti-mouse IgG Spreeta™ surface. Alternatively, a Spreeta™ surface with immobilised proteinA/G can be used for IgG quantification.

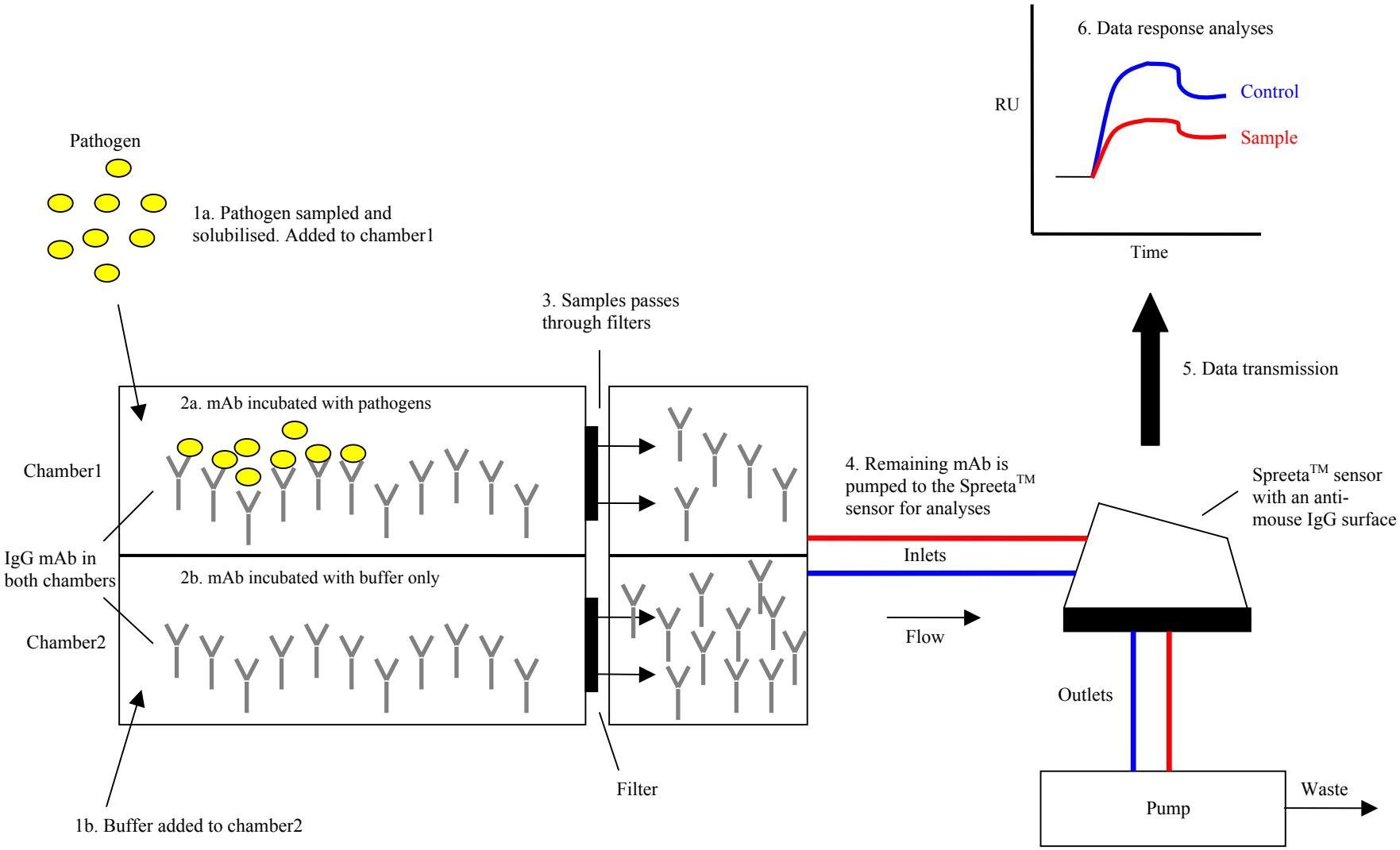

Figure 5.2: Proposed model for future on-site pathogen detection. Please note that the position of the outlets on the drawing is not correct, but is placed at the buttom of the Spreeta™ sensor drawing for simplicity. See text for more detail regarding model setup.

In future plant production, farm sizes will increase and it is therefore crucial for the farm manager to have detailed knowledge of disease afflicted areas. Currently farm managers use agrochemicals when visual inspections of the crop or climatic models suggest pathogen attack. By measuring the concentration of air-borne pathogens it would be possible to predict disease occurance and use agrochemicals in a pre-emptive manner. Precision agriculture systems could detect and quantify specific pathogens in aerial samples and map these disease areas to defined positions within the field. This enables farm managers to perform precise and targeted application of agrochemicals and thereby reduce the pesticide use.

The proposed model-sensor could be connected to air-sampling equipment and be mounted on Autonomous Platforms and Information systems (API), which slowly move around in the field and perform continuous screenings of the field (*5;44*). Researchers envision that the platforms, which are guided by the Global Positioning System (GPS), can identify disease areas and apply patch spraying using onboard spraying systems or map the disease area for manual spraying at a later stage. More developmental work will have to be performed on the described immunoassays before they can be mounted on API systems, but the data presented in this thesis represents a first step towards the implementation of plant pathogen immunosensors on-site.

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